INVESTIGATING THE ANTI-TUMORAL MECHANISM OF AN IMMATURE DENDRITIC CELL TARGETING VACCINE IN COMBINATION WITH 5-AZA-2'DEOXYCYTIDINE AND IFNA IN THE B16F10 MURINE MELANOMA MODEL

by

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A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science

Baltimore, Maryland

May 2021

ABSTRACT

This study investigated the anti-tumoral mechanism of a combination therapy using a DNA vaccine fused to the chemokine MIP-3α, IFNα, and 5-Aza-2'-deoxycytidine in the B16F10 murine melanoma model. Previous data from our laboratory showed enhanced survival in mice treated with this combination therapy, and tumor lysate expression revealed significantly upregulated levels of the chemokine CCL19. Flow cytometric analysis revealed enriched numbers of both vaccine specific and overall CD8+ T cells, as well as the percentage of CD3+ T cells that were CD8+. To further define the protective mechanism elicited by this combination therapy, we challenged female C57BL/6 mice subcutaneously with B16F10 melanoma, and treatment began 5 days later. The vaccine was given intramuscularly by electroporation, with CpG C given intramuscularly two days later at the same site. IFNα was given intratumorally, and 5Aza was given intraperitoneally. Expression of immunologically relevant proteins in the tumor lysate was evaluated by qRT-PCR, and flow cytometric analysis was used to analyze tumor-infiltrating lymphocytes (TILs), dendritic cells (DCs), and NK cells. In line with previous results, we saw reduced tumor sizes in the triple therapy group. Flow cytometric analysis of tumor tissue for this group confirmed previous CD8+ T cell data and indicated increased numbers of DCs and NK cells. Furthermore, the number of CD8+ T effector memory cells were also significantly increased in the combination group. Analysis of tumor lysate also revealed significantly upregulated CXCL13 expression. Additionally, this study also aimed to clarify the role of age and sex in influencing the immune response in the B16F10 model. Older male and female mice

were treated with either vaccine and CpG C (CpG given simultaneously or two days later), CpG alone, or left untreated. Results revealed significantly smaller tumor sizes in females across all groups, in agreement with the literature.

Results from this study suggest that the protective mechanism elicited by the combination therapy group involves the generation of tertiary lymphoid structures (TLS), which have been implicated as a positive prognostic factor in many cancers. However, more experiments, including immunohistochemical staining, must be conducted in order to arrive at a firm conclusion regarding TLS formation.

Primary Reader and Advisor: Richard B Markham

Secondary Reader: James T Gordy

ACKNOWLEDGEMENTS

I would like to thank Dr. Richard Markham for all his guidance as my advisor. His advice and inputs have been incredibly helpful and made this thesis project an enjoyable process. Dr. Markham's constant support, patience, and insights have been critical to my development as a researcher and have encouraged my passion for research.

I would also like to thank Dr. James Gordy for his wonderful mentoring. Working with James was both enjoyable and enlightening, and his teaching has been essential in increasing my knowledge of immunology and cancer.

DEDICATION

This thesis is dedicated to my mother, Dr. Harinder M. Sandhu, and my father, Mr. Davinder Sandhu. None of this would have been possible without your unending love and support. Thank you for everything.

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Introduction

1A. Melanoma

Globally, cancer is the second leading cause of death, and resulted in nearly 9.6 million deaths in 2018. Many of these deaths (over 70%) occurred in low/low-middle income countries with preventable cancers forming a significant number of these cases (1). In the United States, there are expected to be 1.9 million incident cases of cancer in 2021, with over 600,000 deaths. Of these cases, over 100,000 cases will be attributed to melanoma and around 7000 people are expected to die from the disease (2). Melanoma is a skin cancer most common in the white population; the lifetime risk of acquiring the disease is "2.6% for whites versus 0.1% for Blacks, and 0.6% for Hispanics" (3). While the risk rises with increasing age, melanoma is quickly becoming one of the most common cancers in the younger population, especially for those between the ages 35-64 (3). The fatality rate for melanoma tends to be higher than those of other skin cancers (such as squamous cell carcinoma) because it has a higher likelihood of metastasizing to other parts of the body. The metastasis is often the cause of serious illness and death. The 5-year survival rate for stages I and II is over 95%, but later stages have poor survival and high mortality (4).

Treatment for melanoma is dependent on the stage of the cancer; zero stage cancer (melanoma in situ), where the cancerous area is restricted to the top layer of the skin, is usually treated by surgery and excision of the malignant

area, but some doctors may also use topical creams such as imiquimod and radiation (these treatments are rare). First stage cancer, defined by the growth of the melanoma, is also treated by surgery but the excision is often wide and can include the skin around the tumor. Additionally, a doctor may also recommend a “sentinel lymph node biopsy” (SLNB) to scan the lymph nodes proximal to the tumor site for signs of metastasis – spreading of the cancer to secondary organs. If the biopsy finds cancer cells, lymph node removal and/or therapy with an immune checkpoint inhibitor or other targeted drugs is an option. For stage two melanoma, surgery for removal is still performed but as metastasis to nearby lymph nodes is more likely, an SLNB is often recommended. If cancer is confirmed, the route of therapy is similar to that mentioned for first stage cancer. In stage three, the cancer is already present within the lymph nodes upon detection and thus, surgical removal and adjuvant treatment with checkpoint inhibitors and targeted therapy drugs is routine. However, additional therapies including administration of the T-VEC (talimogene laherparepvec) Vaccine, bacille Calmette-Guerin (BCG) vaccine, Interferon alpha (IFNα), or Interleukin-2 (IL-2) directly into the melanoma are also possible; so are radiation therapy and imiquimod cream. The last stage of melanoma, stage four, is diagnosed when the metastasis is pervasive – the melanomas are present within the distal lymph nodes and/or other organs. The skin tumors and lymph nodes are often excised by surgery and treated with radiation; the metastases of internal organs may or may not be removable by surgery. Those that cannot be excised are treated with radiation, targeted therapy, chemotherapy and/or immunotherapy. Options for

those include checkpoint inhibitors and *BRAF/MEK* inhibitors (5,6,7). *BRAF/MEK* inhibitors include drugs such as vemurafenib and are a part of targeted therapy in melanoma. Of the patients presenting with advanced melanomas, nearly 50% have a mutation in the BRAF kinase, where the valine is substituted for a glutamine (104). The use of BRAF and MEK inhibitors have shown rapid antitumor responses, but these responses are often short lived due to tumoral resistance (105). While surgery presents few side effects, administration of immune checkpoint inhibitors requires careful monitoring of the patient as these drugs can cause serious adverse reactions. These therapies primarily include anti-PD 1/PD-L1 (anti-programmed death 1) and anti-CTLA 4 (anti-cytotoxic T lymphocyte antigen-4) antibodies that act on T cells that have been rendered ineffective by the tumor microenvironment. Since these antibodies act on all T cells expressing PD-1 and CTLA-4, the effects are felt throughout the body and include severe nausea, hepatitis, neurotoxicity and myocarditis (6,7). When combined with the use of targeted drug and/or chemotherapy, the side effects are amplified and can prove debilitating for the individual – often requiring hospitalization, as they can be fatal. Similar effects are seen with the administration of IL-2, as well as with *BRAF/MEK* targeted therapy (106).

1A1. Cancer Vaccines

Thus, a treatment that minimizes the side effects while maximizing chances of remission is urgently needed. Vaccines against cancer have historically been unsuccessful; only two licensed cancer vaccines exist on the market – Provenge (Sipuleucel-T) and T-VEC (talimogene laherparepvec)

(discussed in detail later) – and while they are commercially available, they are often implemented as a last resort (8,9). T-VEC is used in the melanoma setting, particularly for late-stage metastatic melanoma unresponsive to first line treatments. Provenge was created based on our knowledge of dendritic cells as potent T cell activators; while its exact mechanism of action is unknown, it works primarily by stimulating T cells via APCs bearing the PAP antigen, which is highly expressed in a majority of prostate cancer cells. It is most often used in cases of advanced prostate cancer that are resistant to hormone therapy (10,11).

The mechanism of T-VEC's action is an oncolytic virus – a virus engineered to kill cancer/tumor cells. Here, the herpes simplex virus, type 1 (HSV-1) is engineered to express human granulocyte-macrophage colony-stimulating factor (GM-CSF), and works primarily by selectively replicating in tumor cells and inducing host immunity. It is injected intratumorally and used in cases of advanced melanoma (11,12). A recent review (11) characterized the efficiency of T-VEC in treating advanced melanoma based on available literature and current clinical trials; in a phase II trial involving patients with stage III and IV melanomas, a response rate of 46.1% was noted in patients that received the T-VEC intratumorally. Additionally, an evaluation of a Phase III trial comparing the efficacy of intratumoral T-VEC vs GM-CSF (granulocyte-macrophage colony-stimulating factor) (the OPTIM trial) delivered subcutaneously in 436 patients presenting with stage III and IV melanomas revealed that T-VEC showed a superior "durable response rate" –

16.3%, whereas GM-CSF was 2.1%. Similarly, the overall response rate reflected a similar trend with T-VEC at 26.4% and GM-CSF at 5.7%. T-VEC also showed better responses regarding lesion reduction (11). Furthermore, the review also compared T-VEC efficacy with other approved melanoma treatments based on 4 clinical trials – the OPTIM trial, two ipilumumab trials (anti-CTLA-4), and 1 vemurafenib (*BRAF* inhibitor) trial. These last three trials involve checkpoint inhibitors and targeted therapy (*BRAF* inhibitors). Overall, T-VEC maintained a significant lead for survival cures when compared to the checkpoint inhibitors – especially for advanced melanomas. It is important to note that while the results are impressive, these analyses were conducted across studies and to show true dominance, a randomized comparative trial featuring the therapies must be performed (11). One of the main agents present in the vaccine, GM-CSF is a cytokine involved in promoting anti-tumoral immunity, but research has indicated that its role maybe more ambiguous than expected as it may instead promote tumor growth as well (11). A literature review examining the role of GM-CSF when administered to advanced melanoma patients in clinical trials analyzed 26 studies; most studies used GM-CSF as an adjunct to other therapy including surgery, peptide vaccines, chemotherapy, etc. No benefit was observed when GM-CSF was used in combination with the peptide vaccines, but the study found "some clinical benefit" in patients who received GM-CSF as an adjuvant in the other settings. However, it is important to note that flu-like side effects were noted (and are also prominent side effects of the T-VEC vaccine) and that

dosing varied across different settings and studies. This review indicated that further studies are clearly required to better define the anti-tumoral role of GM-CSF (13).

Another recent review summarized that the anti-tumoral role of GM-CSF was prominent only when dendritic cells (DCs) were directly involved – as in the case of T-VEC (14). Of note, one study observed tumor *growth* of melanoma when GM-CSF monotherapy was utilized (15). In addition to these questions, its mode of delivery – intratumoral injection – is still uncharted territory for many medical professionals, which curtails its use in the hospital setting. Furthermore, as T-VEC comprises live oncolytic virus, there are biosafety concerns with injecting patients in an "open" area such as a doctor's office – yet another barrier to more frequent implementation (12,13).

Cancer vaccines remain a crucial topic of interest in cancer immunotherapy, with a few promising candidates in clinical trials, including cell-based vaccines such as GVAX, a GM-CSF gene-transfected tumor cell vaccine (16), virus-based vaccines (similar to T-VEC), and peptide vaccines (17). Additionally, advances in systems for optimizing vaccine delivery have also been made in the form of nanoparticles (17), but more research is needed to address gaps in the literature. Finally, "personalized vaccine" strategies have also gained momentum with the discovery of cancer neoantigens – novel antigens expressed by tumor cells that may provide enhanced immunogenicity compared to tumor associated antigens as they are tumor specific (18,19). Since tumor associated antigens are present on

normal cells (albeit are more highly expressed on tumor cells), immune cells are programmed to ignore these signals in an attempt to avoid autoimmunity. This decreases their immunogenic potential and the strength of the antitumoral response. However, tumor specific antigens are capable of bypassing the issue of self-tolerance. Since they are only expressed on tumor cells, these antigens are often immunogenic and can stimulate an effective immune response.

1A2. Interferon Alpha (IFNα) in Cancer Therapy

In addition to vaccines and checkpoint inhibitors, another method of immunotherapy that has been utilized in the cancer setting is recombinant interferon alpha (IFNα). A barrage of studies over the years have evaluated the role of IFNα in a multitude of cancers including melanoma, and most have recorded potent anti-tumoral effects (20,21). IFNα bind its receptor, IFNAR, and triggers anti-cancer activity through multiple pathways. Often, this occurs through potent regulation and enhancement of the immune response, particularly by augmenting DC responses against the tumors and eliciting a Th1 bias in T cells. Th1 T cells promote the proliferation of cell mediated immunity and CD8 cytotoxic T cells. This is especially important as Th1 or cell mediated immunity has shown to be of paramount importance in cancer, as these T cells have cytotoxic potential capable of actively killing cancer cells (22-24).

Furthermore, IFNα2 has been evaluated in the context of melanoma at multiple doses including low, intermediate, and high. As a singular therapy its

effects remain modest, but when used as an adjuvant, its benefits are augmented – it is also the only drug approved by the FDA as an adjuvant therapy. A study examined the positive potential of pegylated IFN α2b (pIFNα) when given weekly for up to 5 years in stage III melanoma patients and found significant improvement in relapse free survival (RFS) 3 years after initiation; however, other factors such as overall survival (OS) and metastasis free survival did not show any significant improvement. It is also important to note that median treatment adherence was 1 year – only 23% of the patient population opted to continue treatment into the 4^{th} and 5^{th} years (25). Overall, multiple studies have reported some level of benefit as seen by OS upon IFNα therapy, but these benefits depend on dosage, delivery method, and duration of treatment. Additionally, IFNα therapy or therapies that encourage a potent IFNα response have been shown to be effective in both murine and human models of melanoma (20-25)

However, despite its benefits, IFNα shows a dose-dependent toxicity with high doses causing severe side effects affecting multiple organ systems and ranging from flu-like symptoms to more serious occurrences such as neurological disturbances and hepatoxicity. The systemic nature of these toxicities is attributed to the abundance of the IFNAR receptor, which is present on a diverse number of cells within the body, and to the high binding efficiency of IFNα to its receptor which amplifies the responses – both negative and positive (26).

1A3. Chemotherapy with 5-aza-2’-deoxycytidine

The final arm of anti-cancer therapy often includes a chemotherapeutic drug. One such agent is 5 aza-2'-deoxycytidine (5Aza, Decitabine), a pan-cancer drug that works as a DNA demethylating agent/methyltransferase inhibitor; it is given as an IV infusion, with dosage dependent on factors such as height and weight (27,28). 5Aza is incorporated into DNA where it interferes irreversibly with the activity of methyltransferases and specifically targets CpG methylated sites on DNA strands. In cancer, loss of DNA methylation is understood as an early event; reduced methylation of prominent genes such as *H-ras and MYC* is highly common in a multitude of cancers. However, increased DNA methylation has also been observed in many cancers – this is especially important when DNA methylation occurs in regions coding for tumor suppressor genes (such as *p53*), which disrupts the control of normal cell division and significantly enhances the chances of a dividing cell going rogue and potentially becoming cancerous. This belief was validated by various studies conducted in the early 2000s; in one study, methylation of at least 1 CpG island covering a tumor suppressor gene was present in over 80% of 600 primary tumor samples covering fifteen different cancers (28). This is one of the main rationales for using 5aza and other demethylating agents. Several experiments have shown reactivation of tumor suppressor function in cancers where the gene was silenced due to DNA methylation (29). The agent does not seem to show any particular specificity and targets a variety of CpG islands, indicating its effect on a large number of targets.

In a study focused on cutaneous melanoma, the researchers found that treatment with 5Aza enhanced recognition of melanoma antigens (including gp100) by melanoma specific CTL via upregulation of MHC Class I antigens (30). Additionally, another study found that 5aza promoted CD8 T cell infiltration, thus augmenting the antitumoral response (31).

1A4. Combination Therapies

The various anti-cancer therapies mentioned above, including vaccines, targeted therapy, chemotherapeutic agents, and Type I IFN, have been investigated both as monotherapies as well in combination with each other. Often, the efficacy of cancer therapy increases significantly when a combination of agents is employed as multiple neoplastic mechanisms are targeted for maximal effect. Since resistance to therapy remains a hallmark of cancer, different types of therapies targeting different mechanisms result in the greatest anti-tumoral effect. This phenomenon is seen in practice in the clinic as well, where physicians use a multi-pronged approach against malignancies including chemotherapy, radiation, immune checkpoint inhibitors, and or surgery (32)

For melanoma, the treatment options have been extensively recorded in the previous part of this chapter. However, late-stage melanoma, which involves extensive metastases, does not respond effectively to current intervention strategies, and has an inordinately high mortality rate (2). For this reason, targeting metastatic melanoma with combination therapy presents an attractive approach. Often, this combination approach deploys a mix of

immunotherapy and chemotherapy, including checkpoint inhibitors against PD-1 and/or CTLA-4 and chemotherapeutic drugs such as Dacarbazine (33). As mentioned above, combination therapies are successful in eliciting a multitude of antitumoral mechanisms. Multiple studies have validated this finding, including work by Lucarini et al, in which the researchers showed enhanced antitumoral efficacy of IFNα and 5aza in a melanoma model. In their study, the therapy increased the presence of CD8+ TILS while decreasing immunosuppressive myeloid cells and Tregs (34). Additionally, studies have also found benefit in combining chemotherapy, radiation, and/or small molecule inhibitors with cancer vaccines (35,36).

1B. Tertiary Lymphoid Structures

Tertiary lymphoid structures (TLS) are a relatively recent phenomenon in the cancer community. Also known as ectopic lymphoid structures/nodes (ELS; ELN), these “tissues” develop in non-lymphoid areas and have been recorded as closely resembling the topography of lymph nodes; they are comprised of lymphocytes (known as tumor infiltrating lymphocytes or TILs) as well as APCs such as follicular DCs, and their occurrence has been documented in a plethora of cancer types including colorectal cancer, melanoma, lung cancer, and breast cancer (37). Owing to the expanding number of studies investigating the role of TLSs in cancer, hypotheses regarding both their prognostic predictive value and immunotherapeutic potential have gained considerable momentum. A major focus of many studies in this area consists of deciphering the anti-tumoral role of TLSs, as well as understanding ways to induce/enhance TLS formation in tumors

(38). While the classical view of an efficient anti-tumoral response involves antigen presentation to B and T cells via DCs in secondary lymphoid organs (SLOs), studies found that these responses also occurred at the tumor site, within organized structures highly similar in both structure and function to SLOs (37,38).

While not unique to cancer, TLSs have received critical attention from the oncology research community. Since they resemble SLOs, they also provide the classical structure for generating an effective anti-tumoral response. This is furthered by a barrage of studies observing positive correlations between the presence of TLS and higher chances of survival/remission in patients with various solid malignancies, suggesting that their anti-tumoral role may possibly be a pan-cancer occurrence (39-41). Mechanistically, TLS formation and function remain areas of research where more information is urgently needed; while a general idea of what is involved in setting up and maintaining these structures does exist, many of the specifics remain unelucidated. However, chemokines and various adhesion molecules have an undeniably critical role in both formation and maintenance of TLS, as well as in TIL recruitment, activation, and proliferation (40,42,45). Some of these chemokines and adhesion molecules include CCL19 and 21, critical for T cell and DC recruitment within SLOs, CXCL13, responsible for B cell recruitment, and addressins such as PNAd expressed by HEVs bordering these TLS. Regarding location, these structures can be present both within the tumor and/or proximal to the tumor; the former being intratumoral TLS, and the latter being peritumoral TLS. Peritumoral TLS

seem to be more common than intratumoral TLS, but the frequency can vary depending on the type of cancer and the tissue involved (42-44).

In melanoma, TLS present an interesting phenomenon. The role of TLS in melanoma has only recently been touched on, and there is an urgent need for more data in understanding the specifics of anti-tumoral TLS function in melanoma. Of the studies that have focused on this topic, most conclude that the presence of TLS – especially in patients with metastatic melanoma – is a favorable prognostic factor indicative of enhanced survival and remission (45,46). However, an understanding of TLS composition regarding effective anti-tumoral cell types, and how TLS can be induced in melanoma to augment current treatment regimens remains an understudied area. Furthermore, while there is a consensus regarding the positive associations of TLS with enhanced survival, only a few studies in recent years have stressed the importance of the cellular components involved in TLS; the cell types involved in these structures have shown to play an important role regarding remission vs relapse in cancer patients. While it remains a nascent field, these findings stress continued investigation in order to develop a more holistic understanding of TLS function in melanoma.

1B1. Evaluation of TLS

Understanding whether a TLS exists in a particular cancer type depends critically on the methods used to evaluate them. Ever since their discovery, deciphering cellular markers indicative of TLS formation have been of utmost importance – as have different methods such as gene expression, flow

cytometry, and histopathology. Since TLS closely resemble SLOs in their organization and cellular composition, a major focus is based on assaying for markers connected to SLOs; these include staining for CD20, CD4, and CD8 in histopathology/immunofluorescence, checking gene expression of B-cell and T-cell related genes (such as *bcl2* and *IL-2*), as well as using flow cytometry to further understand the distribution of cell types within the TLS (44-46)

Arguably, one of the most critical elements of TLS function is high endothelial venule (HEV) expression. HEV are specialized venules present in lymphoid tissues – including secondary lymphoid organs – that provide the requisite scaffolding needed for lymphocyte entry and egress out of lymphoid tissue (47,48). They express 6-sulfosialyl Lewis X ligands (PNAd) that bind the receptor CD62L, which is present on lymphocytes and allows their capture and movement along vessels. A study characterizing the presence of HEV across a range of human solid tumors, including melanoma, found a significant correlation between the density of HEVs present in the tumors and tumor infiltrating CD3+ and CD8+ T-cells as well as CD20+ B-cells. Furthermore, the T cells involved were biased towards Th1 immunity, which has traditionally been implicated in effective anti-tumoral responses and cytotoxic effector functions (49). In line with HEV expression, heightened expression of genes related to lymphocyte homing are also critical indicators of TLS formation, as they support the presence of lymphocytes in the tumor microenvironment (TME). These genes include chemokines such as CCL19,

CCL21, CXCL12, and CXCL13; CCL19 and 21 bind CCR7, which is present on naïve lymphocytes, and CXCL12 binds CXCR4, which has a role in B cell activation and recruitment. CXCL13, which binds CXCR5, has shown to play a critical role in lymphoid organogenesis and B cell trafficking, with studies in deficient mice indicating incomplete maturation of SLOs (44,45). A similar role has been noted for the CCL19/CCR7 axis. Additionally, lymphotoxin beta (LTβ) expression, mediated by activated B cells, has also been shown to sustain TLS formation by feeding into production of the aforementioned chemokines. In concert with this idea, multiple papers have attempted to delineate the most critical chemokines involved in this process; one study identified a “12 chemokine signature” indicative of TLS formation and further indicative of positive prognosis in melanoma. These genes included CCL19, 21, and CXCL13 (46). Furthermore, in addition to lymphocytes, the presence of DCs – especially follicular DCs (fDC) and mature DCs – also plays an important role in both TLS formation and function (50,51). Follicular DCs are a subset of stromal cells present within B-cell follicles and germinal centers and enable effective B cell responses within germinal centers by virtue of antigen presentation and chemokine secretion (52). Their presence has been noted in multiple types of cancer and has been associated with a positive prognosis in most; DC associated markers such as DC-LAMP have shown to be upregulated in tumors having TLS, and high levels of DC infiltration also correspond positively to cytotoxic T cell responses (52).

Thus, when testing for the presence of TLS, it is necessary to take all these factors into consideration. Since it remains a relatively novel field, there is little consensus on what defines the “gold standard” of TLS presence, however, certain characteristics, such as the presence of B and T cells and elevated levels of certain chemokines such as CCL19, are almost a requirement when making the case for presence of TLS formation. Therefore, evaluating a model for TLS formation must involve testing for the factors mentioned in this section – often by multiple methods including qRT-PCR, flow cytometry, and histopathology/immunofluorescence.

1C. B16F10 Murine Melanoma Model

In mice, one of the most used melanoma lines is B16F10, a metastatic cancer cell line derived from female C57BL/6 mice. It presents a well-established and well-studied system in which treatment is extremely challenging. Furthermore, it is considered an accurate model for human melanoma, and thus is an effective model for translational studies (53,54). For the subcutaneous model, a lethal challenge of B16F10 cells (usually $1x10^5$ cells/mouse) is injected subcutaneously into the mouse, and tumor growth is observed within 5-10 days of challenge, with the tumor measuring 1 cm^3 by 14-21 days. Without treatment, after 21 days, the tumors often become necrotic, which, due to humane considerations, requires euthanasia (53). Mice also possess homologues of melanoma antigens expressed in humans including glycoprotein 100 (gp100), and tyrosinase related proteins 1 and 2 (trp1, trp2). Many of these antigens have

been utilized in various studies aiming to promote antitumoral responses in melanoma and therefore present appropriate targets for therapy (55).

1C1. Age and Sex as Variables in the B16F10 Tumor Model

The B16F10 tumor model recently has focused predominantly on young, female C57BL/6 mice; however, sex differences play an important role in progression of the tumor, as well as the immune responses against it. It has been noted that female mice fare better when injected with tumor cells (either subcutaneously or intravenously), as seen by slower tumor growth and a slower rate of metastases. The reasons behind these phenomena have only recently begun being elucidated; Dakup et al noted significantly lower tumor volume and growth rate in females compared to males, and upon investigation found a higher number of CD4+ and CD8+ T cells in females – corresponding to effective antitumoral responses associated with smaller tumor sizes (56).

Data on the effects of age, however, seem to be more conflicting. One study reported that older mice show delayed tumor growth (57), whereas another study investigating the metastatic model claimed that while lung metastases occurred earlier in older mice, the overall tumor growth was slower when compared to the younger cohort (58).

Overall, the limited literature in this subfield clearly indicates age and sex dependent differences in both tumor growth and immune response; more

work is needed to further clarify these discrepancies and the reasons that underlie them.

BACKGROUND

In this study, a DNA vaccine fused to the chemokine MIP-3α and expressing the melanoma antigens Gp100 and Trp2 was given to C57BL/6 mice in combination with IFNα and 5Aza following lethal subcutaneous challenge with B16F10 tumor cells. Previous studies from our laboratory have demonstrated superior efficacy of the antitumoral response of this combination therapy as seen by significantly lower tumor size and increased survival (56). Results revealed enriched T cell and DC populations in the combination group, and interestingly, extremely elevated levels of CCL19 expression. This study aimed to clarify the nature of the antitumoral response initiated by the combination therapy. Based on results from previous experiments, especially those showing enhanced expression of CCL19, and current literature in the field, it was hypothesized that this robust antitumoral response may occur by way of tertiary lymphoid structures, a phenomenon associated with a positive prognosis in many cancers including melanoma. Additionally, age and sex as variables affecting tumor progression in response to treatment were also investigated.

Chapter 2

Investigating the Anti-Tumoral Mechanism of an Immature Dendritic Cell Targeting Vaccine in Combination with 5-Aza-2'deoxycytidine and IFNα in the B16F10 Murine Melanoma Model

2A. Introduction

Cancer immunotherapy has already undergone a remarkable evolution in the last few decades. The discoveries of Interferon, T cells, DCs and NK cells were critical in describing their emerging role in controlling cancer, and more recent studies have built on these initial findings to solidify the indisputable role of the immune system in eradicating tumors (93, 94). The most recent advancements in this field include cancer vaccines and checkpoint inhibitors (94). Checkpoint inhibitors (ICB) such as anti-PD-1 and anti-CTLA-4 have shown good efficacy in a multitude of cancers, especially in those impervious to other modes of therapy such as radiation and/or chemotherapy. Their mechanism of action directly targets inactivated/exhausted T cells by engaging the regulatory molecules CTLA-4 and PD-1, which are upregulated on activated T cells and lead to a reduction in their capability to control and eradicate tumors. Checkpoint inhibitors block the ability of these molecules to interact with their respective ligands, thus allowing T cells to retain their activated phenotype and mount an effective anti-tumoral response (95). However, despite its efficacy, ICB administration is accompanied by a significant number of side effects that can prove to be debilitating (95). Furthermore, while there is increased survival in patients receiving these therapies, most patients still die within 5 years (95).

These issues have motivated more research into other kinds of immunotherapies, including those focused on cancer vaccines and antigen presenting cells (APCs). Only two cancer vaccines have been licensed for clinical use – the T-VEC vaccine and Sipuleucel-T (PROVENGE). Both vaccines use different platforms, with the former using an oncolytic virus and the latter being a cellular vaccine focused on the *ex vivo* expansion of a patients' APCs, especially DCs (10,12). The encouraging safety and efficacy data for cancer vaccines has spurred more research into this field, especially in the arena of DCs.

In the tumor microenvironment (TME), DCs play a critical role. They are the most efficient activators of the T cell response, and the cDC1 subset of DCs is capable of cross-presentation, allowing them to prime CD4+ and CD8+ T cells simultaneously (96). DC activation occurs by of engagement of Toll-like receptors (TLRs), and different DC subsets present differential TLR expression. Historically, IFNα administration as cancer treatment represented one of the first pillars of immunotherapy. Today, IFNα is still a licensed cancer treatment that, when combined other types of treatments, shows enhanced antitumoral efficacy (34). These results led to the belief that the type 1 IFN response played an important role in triggering effective immune responses in the TME, especially those involving T cells. There is extensive literature documenting the role T cells play in controlling and eradicating tumors (97,98), and their efficient activation with tumor antigens is an undeniably important step in the antitumoral response. This efficient activation typically requires involvement of DCs, which phagocytose

antigens, break them down into epitopes, and present them via MHC II pathways (cDC1, pDCs, cDC2) or both MHC I and MHC II (cDC1).

The initiation of the adaptive immune response depends largely on the ability of APCs to uptake antigen and present it to lymphocytes, therefore, therapies that enhance this uptake could potentially show increased antitumoral efficiency. This understanding of the critical role of DCs in the initiation of the adaptive immune response led our laboratory to create a DNA vaccine encoding antigens overexpressed on melanoma cells, Trp2 and Gp100, fused to the chemokine ligand 20 (CCL20), also known as Macrophage Inflammatory Protein-3 (MIP-3α). CCL20 binds chemokine receptor 6 (CCR6), a receptor present primarily on immature DCs (iDCs) (99). Previously published results indicated the enhanced immunogenicity of this vaccine in comparison to one expressing a defective MIP3a fusion, as well as one fused to a non-melanoma antigen (35). Furthermore, previous studies also confirmed that type 1 IFN responses were important in conferring enhanced survival, and to augment these results, direct administration of IFNα was added to the treatment regimen, and CpG C, a known TLR9 agonist and stimulator of Type 1 IFN responses, was chosen as the vaccine adjuvant. Finally, based on the promising potential of combination therapies when employed with cancer vaccines (34), the common chemotherapy agent 5-aza-2'deoxyctidine (5aza) was also added to the treatment. 5aza is a methyl transferase inhibitor that has been shown (119) to overcome inhibition of expression of IFN responsive genes that occurs by methylation, overcoming gene silencing present in many cells. Results for the group receiving the

combination treatment – vaccine, IFNα, and 5aza – showed doubled median survival time and the smallest tumor sizes compared to the negative control. Cellular analysis of the combination group revealed significantly enriched populations of CD3 T cells that were CD8+, as well as the overall numbers of CD8+ T cells per mm^2 of tumor. Furthermore, CD8 T cell presence was also significantly correlated with reduced tumor size, underscoring their critical role in the TME (35).

To further understand the immune responses involved in the triple therapy group, a transcriptome analysis of immune response-related genes was run on the mRNA isolated from the tumor lysate. Results revealed significantly upregulated levels of CCL19, and its expression was 68-fold higher in the triple therapy group than in the IFN+5Aza alone group. Similar to the CD8 T cell data, higher levels of CCL19 were also correlated with a reduced tumor size (35). Chemokine ligand 19 (CCL19) functions primarily as a homeostatic chemokine in secondary lymphoid organs. As a homeostatic chemokine, it is responsible for the recruitment of immune cells into SLOs by binding CCR7, its receptor (59). CCL21, sister chemokine to CCL19, shares this phenotype as well. However, research suggests roles for CCL19 beyond the recruitment of CCR7+ immune cells. Importantly, it has been implicated in the formation of tertiary lymphoid structures (TLS), which are lymphoid aggregates that form ectopically in a multitude of pathological settings including cancer (37). Research regarding TLS is relatively recent, but most studies indicate that their presence is a positive

prognostic factor indicative of an effective antitumoral immune response (37,38,40).

Finally, other parameters that remain largely unexplored in the B16F10 melanoma mouse model are age and sex. The fact that immune responses are significantly impacted by these two variables is now a conclusive finding in most oncology research; however, the literature detailing the specific responses in the context of the B16 melanoma is still scarce. A few studies (56-58) have detailed some of these impacts, but overall, there remains much to be explored in this arena. To begin addressing the gaps in the literature, this study also piloted a small experiment aiming to understand the impact of age and sex in the B16F10 melanoma model. For sex differences, older female and male mice (10-14-weeks-old) were given either vaccine and adjuvant simultaneously, vaccine and adjuvant two days later, adjuvant alone (CpG C), or were given no treatment. The no treatment group was used to understand the differences in natural tumor progression whereas the other groups were utilized to understand differences in responses to therapy. Based on those limited studies, we hypothesized that female mice, despite being older, were likely to outlive the male mice and have slower tumor growth. To understand the impact of age, we used data from females given vaccine and adjuvant two days later and compared them with data from a previous experiment utilizing 6-8-week-old female mice given the same treatment. Here, we hypothesized that older female mice would show slower tumor growth, based on a previous study (57).

Our cumulative findings, especially the marked enhancement of CCL19 expression, led us to hypothesize that the formation of TLS may contribute to the enhanced survival observed in the triple therapy group. The current study aimed to explore this hypothesis by evaluating TLS formation in the triple therapy group by way of flow cytometry and qRT-PCR analysis. Furthermore, as age and sex have been identified as important variables impacting the immune response, this current study also aimed to explore the variations in tumor growth and differences in response to therapy between male and female mice, as well as young and old female mice.

2B. Results

2B1. Tumor Size Differences between Administered Therapies

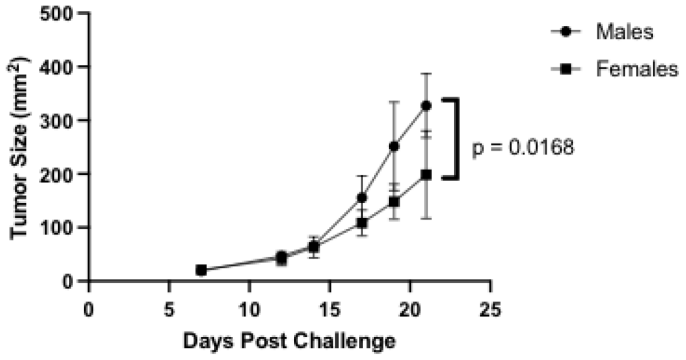
This current study covered two different experiments, and tumor size measurements were taken for both.

First, to understand potential differences in tumor growth and response to treatment mediated by sex differences, 10-14-week-old male and female C57BL/6 mice were used. Mice were subcutaneously challenged with a lethal dose of B16F10 melanoma cells ($5x10^4$), and tumor sizes were measured every 1-3 days using automated calipers. Mice were challenged on day 0, and vaccinated (if indicated) on days 5, 12, and 19; adjuvant was given on days 7, 14, and 21 (if indicated). The no treatment group was used to compare differences in tumor growth between older male and female mice, and the efficacy of CpG C monotherapy was also evaluated – as were

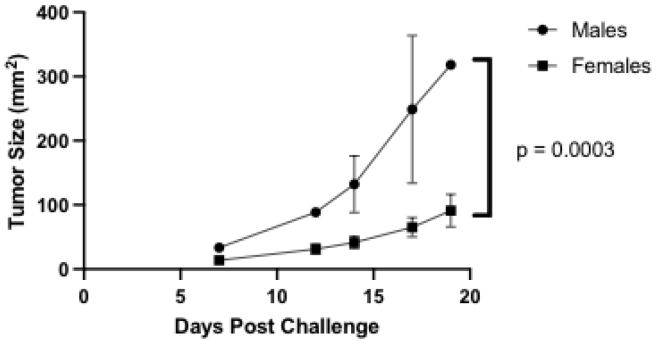
differences in delivering vaccine and adjuvant simultaneously v/s adjuvant given 2 days post vaccination. Two groups given adjuvant at two different timepoints were utilized as previous data from the laboratory indicated greatest production of cancer antigens 2 days post vaccination; in order to validate this finding and explore any possible differences, vaccine and adjuvant were also given simultaneously to a separate group of mice. Statistical analysis of these differences was performed using a 2-way ANOVA or a mixed effects model if the data did not meet the assumptions of the ANOVA. Results indicated that across all groups, including no treatment, female mice had significantly smaller tumors (Fig. 1a-d), and some statistical significance was established between male and female mice. Group II females (those receiving vaccine and CpG separately) also saw the longest survival (euthanized day 30) relative to the other groups. Importantly, males across all groups were euthanized by day 23. Interestingly, for the males, vaccine and adjuvant given simultaneously (Group I) worked more efficiently; the average size of tumors for Group 1 were around 150 mm^2 (Fig. 1e) and differences between the therapy and no treatment were highly significant ($p = 0.0003$). The group given vaccine and CpG C two days later had an average tumor size of 250 mm^2, also significantly different compared to the no treatment group ($p = 0.0369$) (Fig. 1b). Overall, female mice saw significantly smaller tumors and responded better to therapy – especially to vaccine and CpG C given two days later (Fig. 1b). These findings also validated previous results. To understand the impact of age on tumor growth and response to

treatment, tumor sizes from older female mice utilized in this experiment were compared to a previous experiment involving 6-10-week-old female C57BL/6 mice (Fig. 1f). Since only Group II (vaccine and CpG given 2 days later) was consistent across the two experiments, it was the only one used for analysis. A 2-way ANOVA revealed no significant differences between older and younger female mice receiving the vaccine.

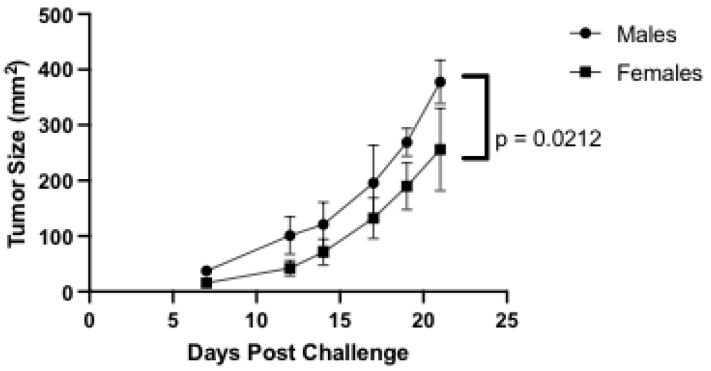
a)



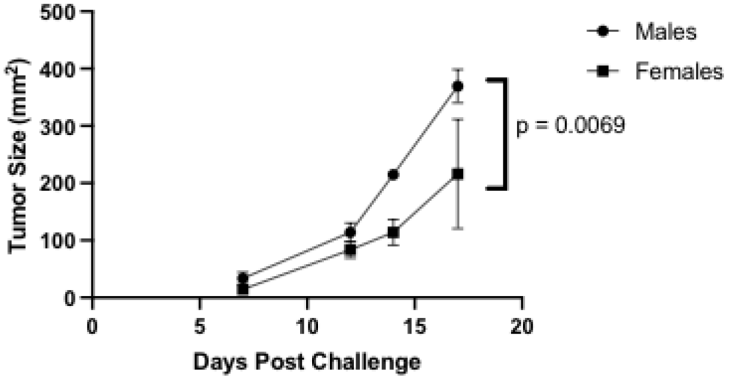
b)



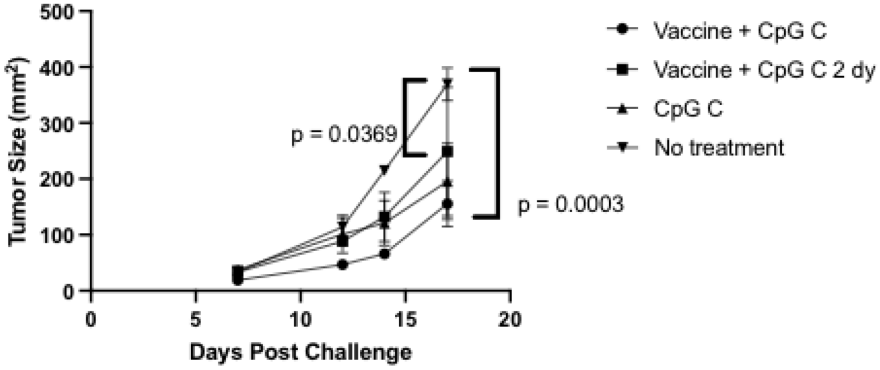
c)


Tumor Size (mm²)
Days Post Challenge
Males
Females
p = 0.0212

d)


Tumor Size (mm²)
Days Post Challenge
Males
Females
p = 0.0069

e)


Tumor Size (mm²)
Days Post Challenge
Vaccine + CpG C
Vaccine + CpG C 2 dy
CpG C
No treatment
p = 0.0369
p = 0.0003

f)

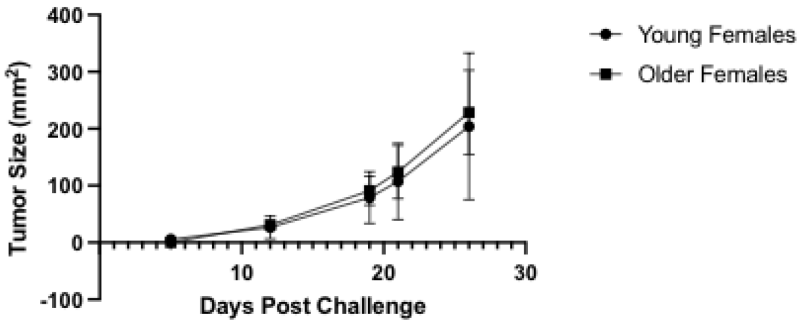
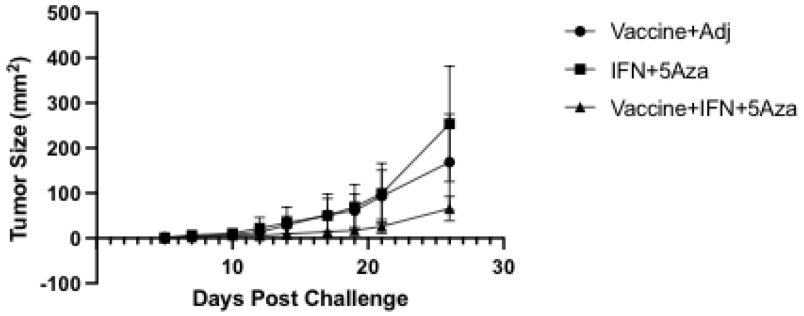


Fig. 1. **Tumor size variability based on age and sex differences.** a-d) Differences in tumor growth in older female mice v/s older male mice; these data represent one experiment with n = 3 F and 2 M per group. Each graph stratifies the differences between males and females based on the treatment indicated: a) Vaccine + CpG given simultaneously, b) Vaccine + CpG given 2 days later, c) CpG alone, and d) no treatment. e) Differences in tumor growth between groups for male mice; same experiment as a-d. f) Differences in tumor size between older and younger female mice based on Vaccine + CpG given 2 days later.

Second, tumor sizes were evaluated in the TLS experiment in the same manner. For this study, 6-10-week-old female C57BL/6 mice were utilized and challenged the same manner as mentioned above. This experiment also included administration of IFNα and 5Aza (if indicated). Results from this study corroborated previously published data as tumor sizes in the triple therapy group were the smallest (Fig. 2a); it is important to note that due to the low power of the experiment, statistical significance could not be established between groups for this experiment, but the combination of 3 experiments including the current study did show significant differences between therapies (Fig. 2b).

a)



b)

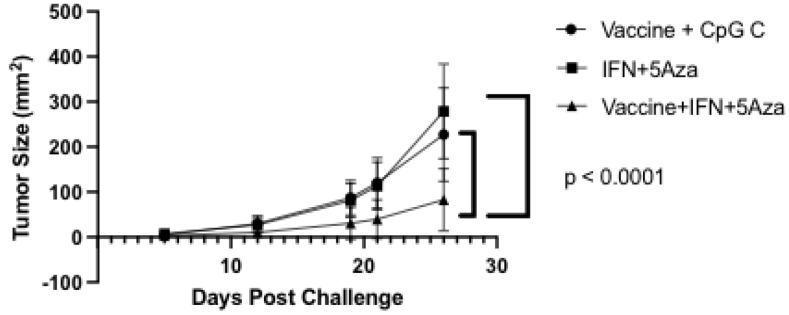


Fig. 2. **Tumor growth in response to therapy.** Vaccine was given intramuscularly on days 5, 12, and 19 post challenge, IFNα was given subcutaneously at the tumor site and 5Aza was given intraperitoneally opposite to the tumor site. Panel a) depicts the data from one experiment and b) is a combination of data from 3 experiments.

2B2. Tumor Lysate Gene Expression

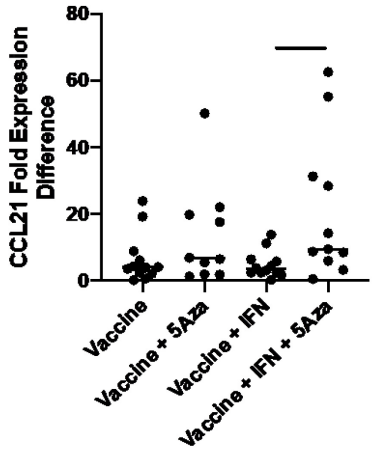
Since previous results indicated enriched levels of CCL19 and the role of the type 1 IFN response in enhancing survival in the combination group, additional genes related to these scenarios were chosen for transcriptional assessment. As the sister chemokine to CCL19, CCL21 was the first gene evaluated. Since both bind CCR7 and share a largely redundant phenotype, it was hypothesized that CCL21 expression was most likely to mirror CCL19

expression. However, surprisingly, this was not the case. CCL21 expression was not upregulated to the same extent between the treatment groups, and neither was there any correlation between its presence and tumor size (Fig. 3a and b). In order to explore this in further detail, we focused on genes involved in the upregulation in CCL19. This led us to genes related to the Type 1 IFN pathway, as interferon related genes have been implicated in CCL19 expression (100,101). Six genes were chosen for this panel and included *IRF3, IRF9, IFNB, IFNA, RelB*, and *MB21D1* (Fig. 3c). All primers were purchased from Applied Biosystems, and qRT-PCR was run on late timepoint RNA extracted from previous experiments. Gene expression was highly variable across all candidates and no genes were either significantly upregulated or downregulated for any particular group.

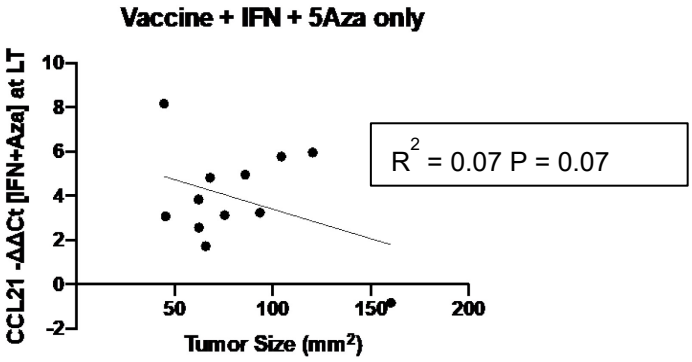
Based on these results, we then tested for genes related to TLS formation. As CCL19 has been found to be significantly upregulated in solid tumors presenting with TLS, we assessed the expression of two genes, *CXCL13* and *CD62L/LSEL.* Both genes have are involved in TLS formation; CXCL13 is a chemokine responsible for B cell trafficking into SLOs by binding CXCR5 (102), and CD62L is an integrin upregulated on lymphocytes capable on “rolling” on high endothelial venules (HEVs) (49). Both lymphocytes and HEVs have been implicated as critical components of TLS (35,49,102,103). CXCL13 expression was significantly upregulated in the triple therapy group, especially in comparison to IFN + 5Aza (Fig. 3d), and its presence was also significantly correlated with reduced tumor size (Fig. 3e). Comparison was done based on both FED as well

as ΔCt values; for the FED, both the vaccine and the triple therapy group were compared to IFN + 5Aza alone whereas ΔCt values were compared across all groups. Based on FED and ΔCt values, LSEL/CD62L was also upregulated (Fig. 3f) and was significantly upregulated in the combination group when compared to IFN + 5Aza. However, there were no significant differences between vaccine alone and the combination group.

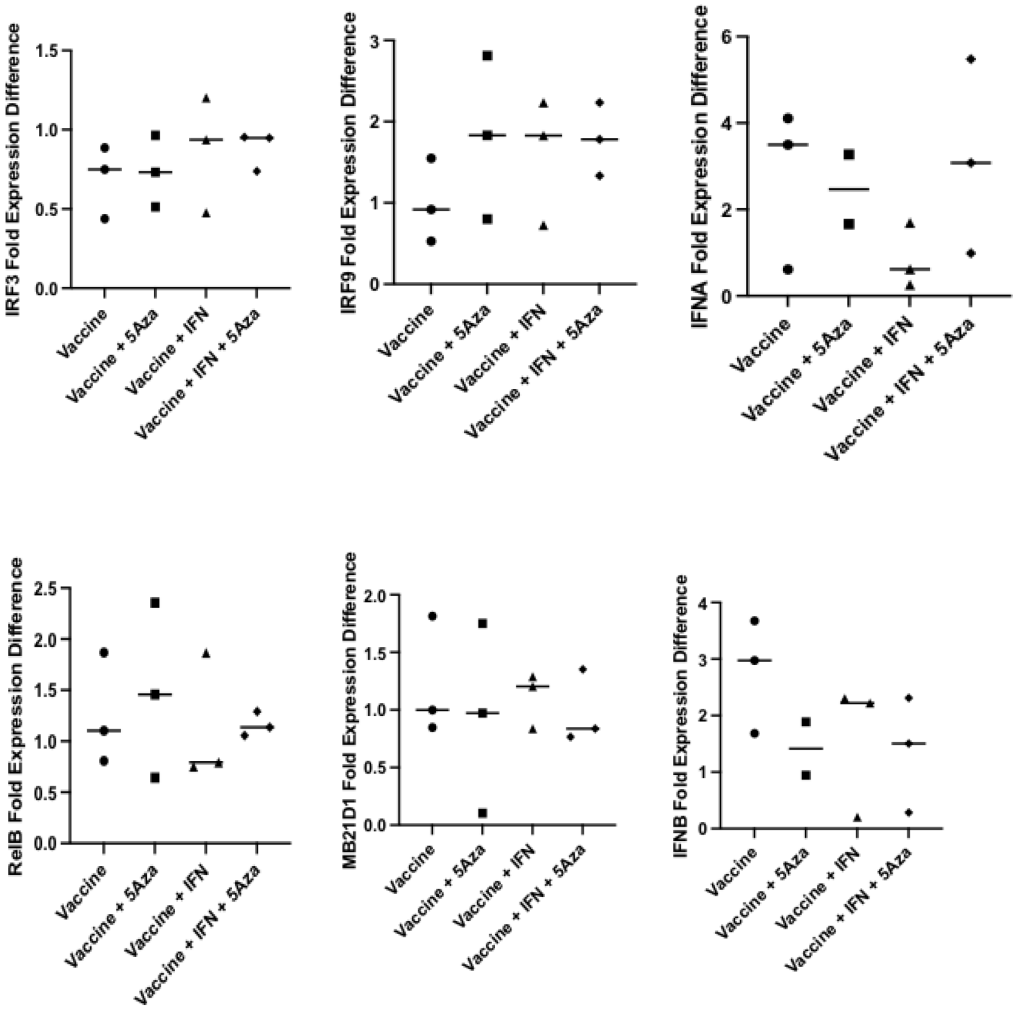
a)



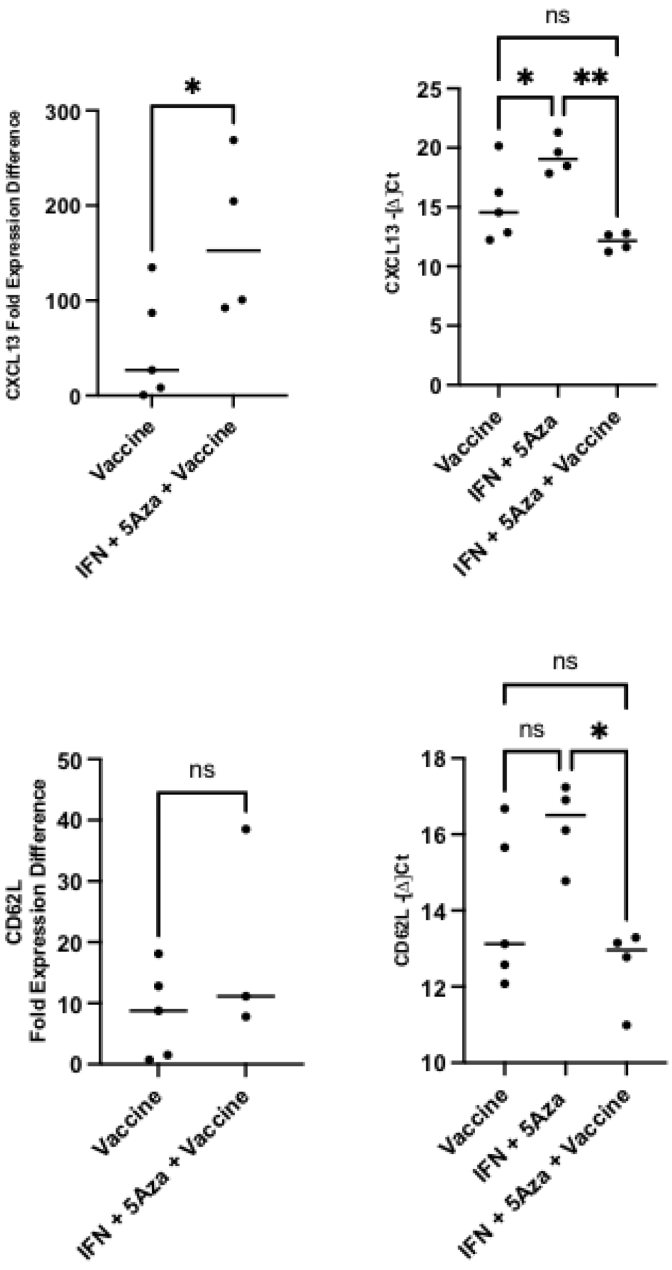
b)



c)


IRF3 Fold Expression Difference
1.5
1.0
0.5
0.0
Vaccine
Vaccine + 5Aza
Vaccine + IFN
Vaccine + IFN + 5Aza
IRF9 Fold Expression Difference
3
2
1
0
Vaccine
Vaccine + 5Aza
Vaccine + IFN
Vaccine + IFN + 5Aza
IFNA Fold Expression Difference
6
4
2
0
Vaccine
Vaccine + 5Aza
Vaccine + IFN
Vaccine + IFN + 5Aza
RelB Fold Expression Difference
2.5
2.0
1.5
1.0
0.5
0.0
Vaccine
Vaccine + 5Aza
Vaccine + IFN
Vaccine + IFN + 5Aza
MB21D1 Fold Expression Difference
2.0
1.5
1.0
0.5
0.0
Vaccine
Vaccine + 5Aza
Vaccine + IFN
Vaccine + IFN + 5Aza
IFNB Fold Expression Difference
4
3
2
1
0
Vaccine
Vaccine + 5Aza
Vaccine + IFN
Vaccine + IFN + 5Aza

d)


CXCL13 Fold Expression Difference
300
200
100
0
*
Vaccine
IFN + 5Aza + Vaccine
CXCL13 -[Δ]Ct
25
20
15
10
5
0
ns
*
**
Vaccine
IFN + 5Aza
IFN + 5Aza + Vaccine
CD62L
Fold Expression Difference
50
40
30
20
10
0
ns
Vaccine
IFN + 5Aza + Vaccine
CD62L -[Δ]Ct
18
16
14
12
10
ns
ns
*
Vaccine
IFN + 5Aza
IFN + 5Aza + Vaccine

e)

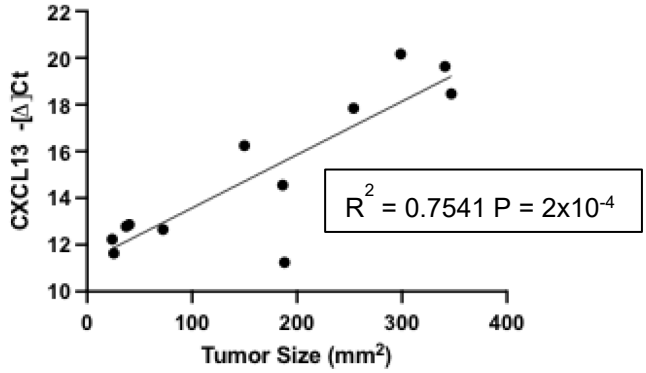


Fig. 3. **qRT-PCR analysis of tumor RNA.** a) Fold expression difference of CCL21 relative to the IFN+Aza group (outliers removed) and b) Correlation between CCL21 -dΔCt values and tumor size. CCL21 expression was analyzed for 3 independent experiments and correlation for 1. n = 3 - 5 mice per group. c) Fold expression of six candidate genes (IRF3, IRF9, IFNB, IFNA, RelB, and MB21D1) relative to IFN+Aza expression. n = 3 mice per group, data from 1 experiment. d) – f) Fold expression and ΔCt values of two candidate genes (CXCL12 and LSEL) were analyzed; for FED, analysis was performed using the IFN+Aza group as the comparator. n = 3 mice per group, data from 1 experiment. e) Correlation analysis between CXCL13 expression and tumor size.

2B3. Tumor Infiltrating Lymphocytes, DC, and NK Analysis

To understand the immune makeup of the TME, flow cytometric analysis was performed on tumor tissue. Prepation of tumor cells, staining, and gating were performed as indicated in sections 2C3 and 2C4. First, the presence of DCs was evaluated. The log tranfsformed counts of overall Cd11c + cells and Cd11c CD8a+ cells were significantly upregulated in the combination therapy group, and %Cd11c cells that were CD8α+ were trending higher in the combination group relative to both vaccine and IFN+5aza (Fig. 4a). Importantly, this same subset of cells was also correlated with reduced tumor size, as indicated by an R^2 of 0.33, albeit with a trending p value (p = 0.067) (Fig. 4b). These results align with previous published (36) and unpublished results. Since this experimental model involves IFNα and

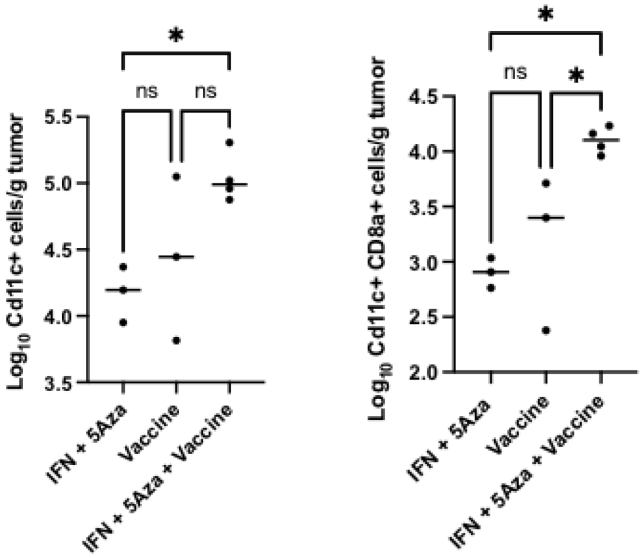
previous results stressed the role of type 1 IFN responses in conferring protection, this study also aimed to clarify the presence of pDCs. pDCs, when activated, produce large amounts of IFNα and can activate CD8+ T cells without CD4+ T cell help. While not significantly different between groups, pDCs were elevated in the triple therapy group ($p = 0.0972$). T cell inflitration into the TME was also evaluated in the same manner as DCs. In this panel, CD8+ T cells (CD3 CD8+) were examined to ensure reproducability of previously published data, and the CD8+ memory effector (Tem) population was evaluated based on its critical antitumoral role. Similar to DCs, the log transformed numbers of both CD3+CD8 T cells and Tem T cells (CD3+CD8+ CD62L-CD44+) were signficantly upregulated in the combination group relative to IFN+5aza (Fig. 4c) with a $p < 0.05$, and the memory subset was significantly upregulated compared to vaccine as well. Additionally, both T cell subsets were also highly correlated with reduced tumor size, as indicated by an R^2 of 0.5729 and a $p = 0.0113$ for CD8 T cells, and an R^2 of 0.8146 and a $p = 0.0004$ for the effector memory subset (Fig. 4d).

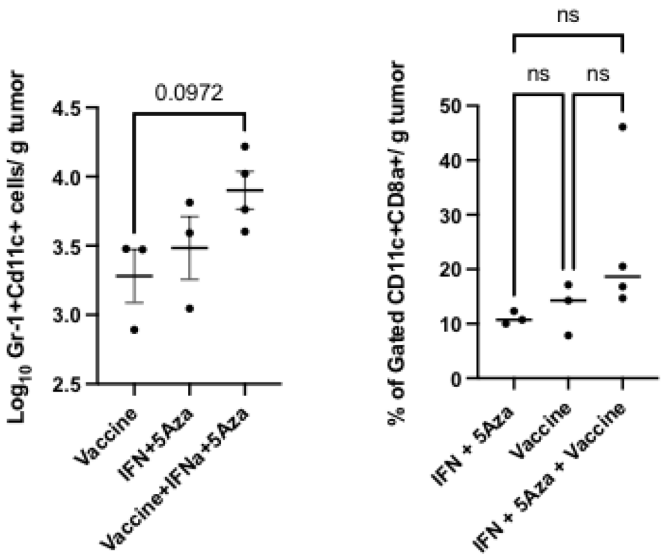
In contrast to both DCs and T cells, staining for B cells in the tumor tissue did not yield similar results. B cells were evaluated for infiltration into the TME using B220 and CD19 as B cell markers. While the analysis revealed no significant differences in the CD19+ population between treatment groups, B220+CD19+ double positive B cells were significantly lowered in the combination group when compared to vaccine alone (Fig. 4e).

Furthermore, there was no correlation between CD19+ cell positivity and reduced tumor size (Fig. 4f).

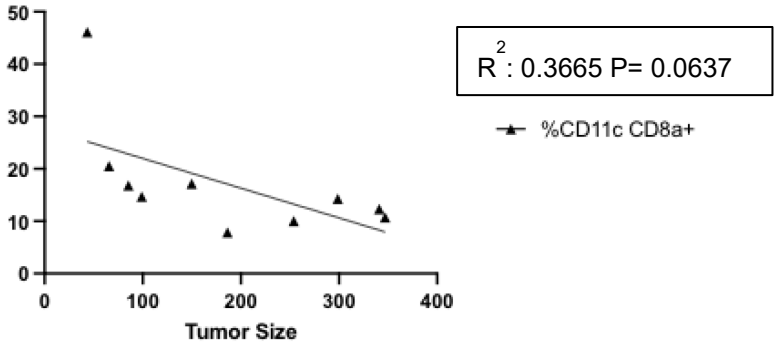
Last, in addition to the other cell types, NK cells were also evaluated for presence in the tumor tissue. NK1.1 was used as the marker for NK cells. Analysis revealed a significantly higher population in the combination group compared to vaccine alone as well as an upwards trend compared to IFN+Aza group (Fig. 4g). NK cell infiltration was also significantly correlated with reduced tumor size (Fig. 4h).

a)

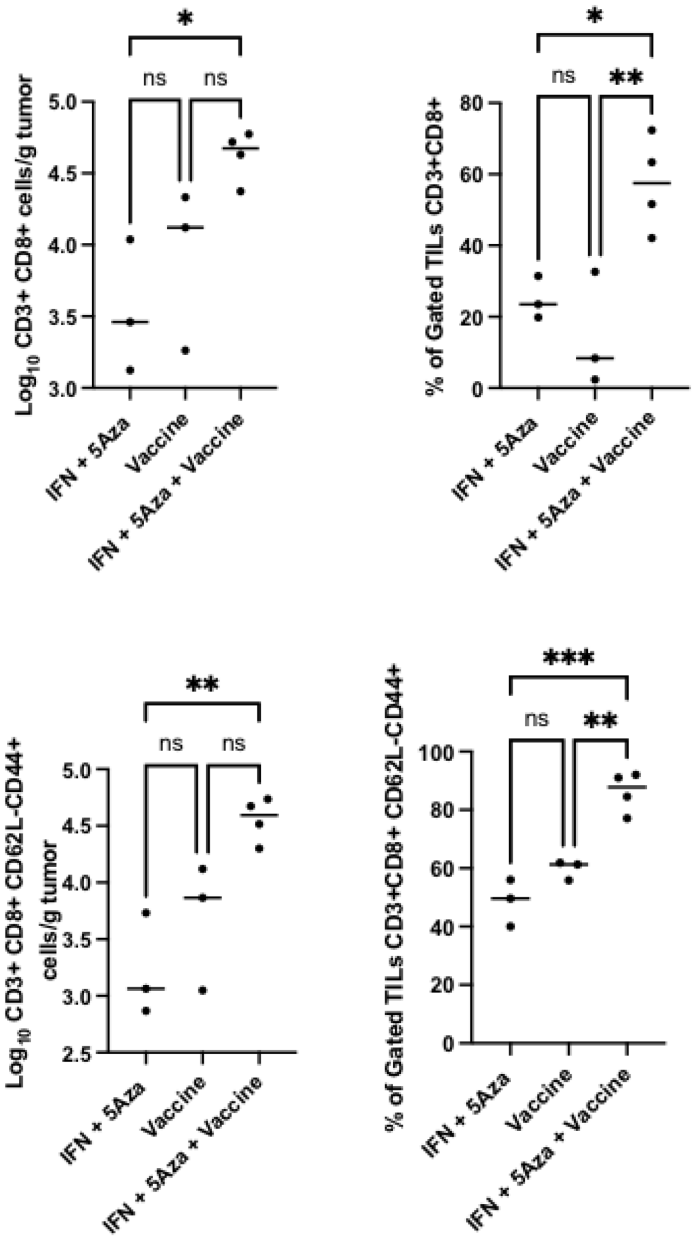



0.0972
Log10 Gr-1+Cd11c+ cells/ g tumor
4.5
4.0
3.5
3.0
2.5
Vaccine
IFN+5Aza
Vaccine+IFNa+5Aza
ns
ns
ns
% of Gated CD11c+CD8a+/ g tumor
50
40
30
20
10
0
IFN + 5Aza
Vaccine
IFN + 5Aza + Vaccine

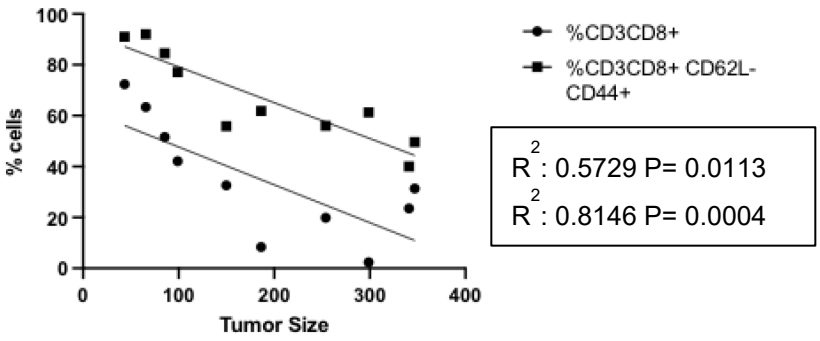
b)


50
40
30
20
10
0
0
100
200
300
400
Tumor Size
R2: 0.3665 P= 0.0637
%CD11c CD8a+

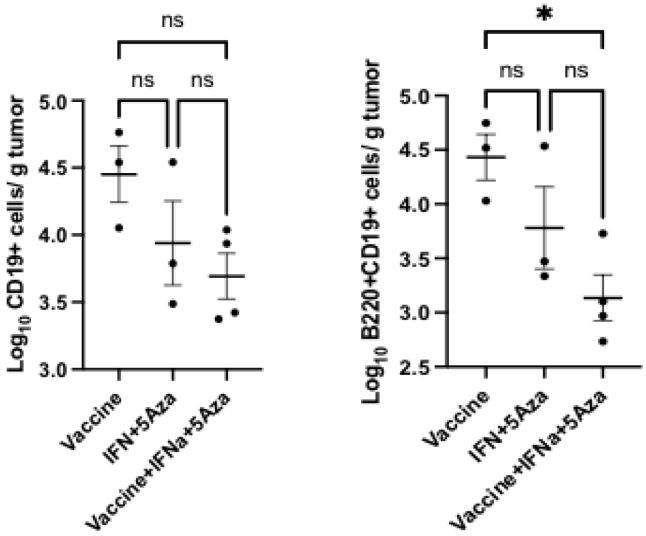
c)


*
ns
ns
Log10 CD3+ CD8+ cells/g tumor
5.0
4.5
4.0
3.5
3.0
IFN + 5Aza
Vaccine
IFN + 5Aza + Vaccine
*
ns
**
% of Gated TILs CD3+CD8+
80
60
40
20
0
IFN + 5Aza
Vaccine
IFN + 5Aza + Vaccine
**
ns
ns
Log10 CD3+ CD8+ CD62L-CD44+ cells/g tumor
5.0
4.5
4.0
3.5
3.0
2.5
IFN + 5Aza
Vaccine
IFN + 5Aza + Vaccine
***
ns
**
% of Gated TILs CD3+CD8+ CD62L-CD44+
100
80
60
40
20
0
IFN + 5Aza
Vaccine
IFN + 5Aza + Vaccine

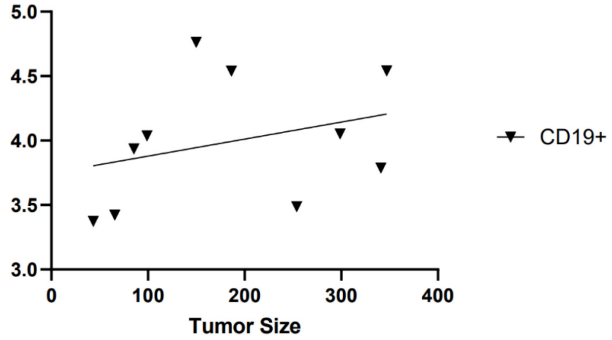
d)


%CD3CD8+
%CD3CD8+ CD62L-CD44+
% cells
Tumor Size
R2: 0.5729 P= 0.0113
R2: 0.8146 P= 0.0004

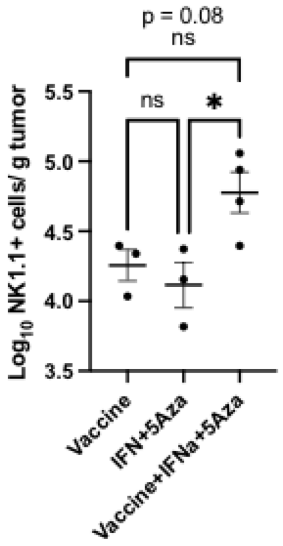
e)


ns
ns
ns
Log10 CD19+ cells/ g tumor
Vaccine
IFN+5Aza
Vaccine+IFNa+5Aza
*
ns
ns
Log10 B220+CD19+ cells/ g tumor
Vaccine
IFN+5Aza
Vaccine+IFNa+5Aza

f)


CD19+
Tumor Size

g)



h)

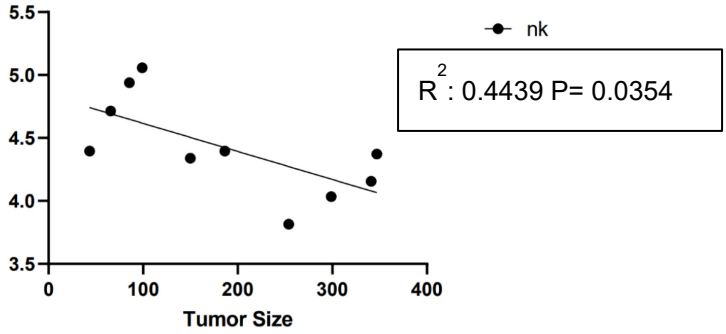


Fig. 4. **TILs, DC, and NK analysis**. a) Flow data showing log transformed numbers of CD11c and CD11c+CD8a+ cells, as well as percentage of CD11c+CD8a+ cells. b) Correlation between CD11c+CD8a+ cells and tumor size. Data from 1 experiment, n= 3 mice per group. c) Flow data showing log transformed numbers of CD3+ CD8 T cells and Effector Memory CD8 T cells, as well as percentage of CD3+ cells that are CD8+ T cells, and percentage of CD3+CD8+ T cells that are CD62L- and CD44+. d) Correlation between the two T cell subsets and tumor size; n = 3 mice per group and data from 1 experiment. e) Flow data showing log transformed numbers of both B220 and CD19 DP B cells as well as CD19+ B cells. f) Correlation between CD19+ cells and tumor size; n = 3 mice per group and data from 1 experiment. g) Flow data showing log transformed numbers of NK1.1+B220- cells h) Correlation between NK cells and tumor size; n = 3 mice per group and data from 1 experiment.

2C. Materials and Methods

2C1. Tumor Model

Age and Sex Experiment

10–14-week-old female and male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were challenged subcutaneously in the left flank on Day 0 with $5x10^4$ cells B16F10 tumor cells (>95% viability, volume of 100 µl). Tumor size was taken every 1-3 days (units recorded were square mm); endpoint analyses were to assess differences in tumor growth and survival between males and females. Mice were kept in the study until they died or had to be sacrificed. Conditions for humane sacrificing of the mice included tumor size exceeding 20 mm, tumor tissue necrotizing, and significant lethargy (59).

Combination Therapy Experiment

6–10-week-old female C57BL/6 mice were challenged in the same manner as for the age and sex experiment. Tumor size was recorded every 1-3 days in square mm, and endpoint analyses were to assess presence and/or differences in TLS formation in the group receiving the vaccine, IFNα, and 5Aza. The same criteria for sacrificing mice were used in this experiment as mentioned above.

2C2. Vaccine Design, Vaccination, and Therapeutics

2C2i. Plasmid Design

The plasmid utilized for this vaccine was a modified pCMV vector (36) and encoded the mouse melanoma antigens gp100 and trp2 fused to the mouse chemokine MIP-3α. Additionally, the secretion signal from

mouse IP-10 was also included. The plasmid was extracted from DH5-a *E. coli* using Qiagen Endo-Free Plasmid Maxi and Giga kits. Post extraction, vaccine purity was measured by Nanodrop spectrophotometry and gel electrophoresis, and the sequence was confirmed by restriction enzyme analysis as well as complete DNA sequencing. Restriction enzyme analysis included enzymes XhoI and XbaI (NE Biolabs).

2C2ii. Vaccinations

Extracted DNA was diluted in 1x endo-free PBS at 1 mg/ml, and each mouse received a 50-µg dose intramuscularly (volume of 50 µl) in the right tibialis. Each vaccination was followed by in vivo electroporation. Pulses were delivered with an ECM 830 Electro Square Porator™ with 2-Needle Array™ Electrode (BTX Harvard Apparatus®; Holliston, MA) using the following parameters: 106 V; 20 ms pulse length; 200 ms pulse interval; 8 total pulses. Based on previous published data, CpG C (ODN2395) was also given intramuscularly as an adjuvant at the same dosage and site; it was also diluted to 1 mg/ml in 1x endo-free PBS. The vaccine was given at days 5, 12, and 19 post challenge, and adjuvant was given 2 days after each vaccination.

2C2iii. Therapeutics

Two additional therapies (if indicated) were also utilized. These included Recombinant Mouse Interferon Alpha-A (IFNα) and 5 Aza 2'-deoxycytidine (5aza). IFNα was delivered intratumorally as a high dose

(10,000 units) followed by 3 days of low doses (1000 units), whereas 5aza was given intraperitoneally at 1 mg/kg. Both therapies were given as 50 µl volume to each mouse at the described timepoints as indicated in Supp. Fig. 4.

2C3. Lymphocyte and Tumor Infiltrating Lymphocytes Isolation (TILS) and Flow Cytometry

2C3i. Lymphocyte Isolation – Spleen

Cell suspensions of each were made under sterile conditions by grinding harvested tissue with pestles and filtering them through sterile 60 uM mesh filters. The suspensions were spun down at 4 deg C, 250 g for 7 minutes, and spleens were processed by the addition of ACK lysis buffer to lyse RBCs (1 ml/spleen, incubated at RT for 3-5 mins) and washed with sterile PBS (20-30 ml to stop lysis); cells were spun again at the same settings and were either resuspended in sterile 1x PBS/FACS buffer for immediate staining or 4 ml freezing media/spleen for cryostorage.

2C3ii. TILs Isolation – Tumors

Tumor suspensions were made by grinding tumor tissue with frosted ends of microscope slides, washing with sterile PBS, and filtering through sterile 60 µM mesh. ACK lysis buffer was also added to the tumor suspensions to lyse RBCs. Cells were resuspended in sterile 1x PBS and counted using a ZI Coulter Counter and used for subsequent staining.

2C4. Flow Cytometry

Tumor cells resuspended in 1x PBS were transferred to a 96 well V-bottom plate and stained with a live/dead (L/D) stain (30 mins at RT in the dark) and washed with FACS buffer (0.5% BSA in sterile 1x PBS). Following L/D, cells were stained with a surface antibody cocktail (20 mins at RT in the dark) and read on the Attune NxT flow cytometer. Gating strategy was as follows: live singlets were gated based on L/D stain, and then were stratified based on the different stains used. For Panel 1, live singlets were sorted into B220+ cells and further separated based on expression on Cd11c and Gr-1 (pDC) and CD19 (B cells). For B220- cells, cells were selected based on NK1.1 expression (NK cells), as well as Cd11c and CD8a expression indicative of dendritic cells. For Panel 2, live singlets were sorted into CD3+ and CD3- populations. From the CD3- population, cells were gated on CD11c and CD8a expression (DCs). The CD3+ population was used to gate for T cells; it was further stratified into CD8+ cells. The CD3+ CD8+ gated population was analyzed for CD44 and CD62L expression. A quadrant gate created for these populations, and the CD3+CD8+CD44+CD62L-population was also further gated for CCR7 expression. Flow data was analyzed using Flow Jo software and total cell counts were back calculated from tumor weight and volume of cells plated. Multiple panels were performed on the harvested tissue; tumors were stained to look for various immune cells as elaborated below.

2C4i. Panel I – DCs and B220+ cells

Stains used: B220-FITC, CD19-PECy7, Gr-1-APC, NK1.1-AF700, and CD11c-PE

2C4ii. Panel II – CD8+ T effector memory cells

Stains used: CD3-PerCpCy5.5, CD8-FITC, CD62L-APC, CD44-AF700, CCR7-PECy7, and CD11c-PE

2C5. Gene Expression by qRT-PCR

Gene expression was analyzed by qRT-PCR on RNA extracted from previous experiments (experiments were performed using the same parameters set in the current study) as well as the current experiment. RNA extraction was performed using Trizol, the high-capacity cDNA synthesis kit was used to reverse transcribe mRNA to cDNA, and gene expression was analyzed for the late timepoint. Probes used were GAPDH, CCL19, CCL21, RelB, IRF3, IRF9, MB21D1, IFNA, IFNB, CD62L, and CXCL13 and the procedure was performed using TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assays. To calculate ΔCt, the Ct value of the gene of interest (e.g., CXCL13) is subtracted from the Ct value of the housekeeping gene (GAPDH). Similarly, ΔCt was calculated by subtracting the Ct values of the gene of interest from the Ct value of the IFN+5Aza group. Finally, fold expression difference (FED) was calculated by applying the following formula: $2^{(-\Delta\Delta Ct)}$. Analysis was performed in Microsoft Excel.

2C6. Statistical Analysis of Data

For tumor sizes, qRT-PCR, and flow cytometric analyses, a one-way ANOVA was used to test for significance. Tumor sizes taken at one time point were log2 transformed, and tumor growth was analyzed by two-way ANOVA when data were being compared across multiple time points. If the data were not uniform (e.g.: one group had only 3 mice whereas the others had 4), Mixed Effects Analysis was used instead of the two-way ANOVA which requires complete data uniformity. Scatter plot correlations between tumor sizes and markers of interest were analyzed by simple linear regression. All data was stored in Microsoft Excel, and Prism Graphpad 9 was used for both statistical analyses and creation of figures. For all datasets, the significance level was set to $p \leq 0.05$.

Chapter 3

Discussion

3A. Age and Sex as Variables in the B16F10 Melanoma Model

Historically, age and sex as variables affecting the immune response have largely been disregarded in the research setting; only recently have questions surrounding these variables gained traction and received a great degree of interest from the oncology community. As previous studies with our B16F10 subcutaneous melanoma model have been consistently performed in 6-10-week-old female C57BL/6 mice, the current study aimed to understand and expand on any possible differences in tumor progression, response to treatment, and survival between male and female mice, as well as young v/s older female mice.

3A1. Sex Differences

To determine sex differences, twenty 10-14-week-old female and male C57BL/6 mice were separated into 4 groups; Group I received the vaccine and adjuvant simultaneously, Group II received vaccine alone with adjuvant given 2 days after vaccination, Group III only received adjuvant, and Group IV received no treatment; each group consisted of 3 females and 2 males. All males had to be euthanized by day 23 as they had surpassed the set thresholds. Importantly, both males in the no treatment group were euthanized on day 17, whereas the females in the group were euthanized on day 21. As Group IV received no treatment, this group was used as a marker for natural progression of the subcutaneously implanted tumor. Previous

studies (56-58) have indicated that tumor progression is slower in females compared to males and our pilot study aligns with these findings. However, since the power of the experiment remained small, the experiment will have to be repeated in order to confirm these initial observations.

Additionally, this study design also aimed to clarify the response generated by CpG C, the adjuvant. Previous experiments from our lab have utilized CpG C based on the prominent involvement of IFNα in the antitumoral response (59) and the observation that CpG C is a potent activator of IFN related immune responses (82). However, the efficacy of CpG C alone in this model had not been evaluated; this is important as studies by others have indicated an antitumoral response attributed to singular therapy with CpG C (78). Therefore, to clarify the extent of CpG C's involvement in the immune response, Group III was treated with adjuvant alone; CpG was given intramuscularly on days 7, 14, and 21 post challenge at the same dosage (50 µg, volume of 50 µl) into the right tibialis. On day 21, both males and one female were euthanized as their tumors had surpassed 20 mm^2 in diameter, and the remaining two females were euthanized on day 23. These data, especially in the females, potentially indicate that while CpG C does provide some level of protection, it is relatively modest. Thus, the boost in survival we see with the vaccine and CpG C is not solely attributable to adjuvant alone – the combination of both is critical.

Furthermore, previous published results from our lab showed greatest production of vaccine antigens 48 h post transfection in HEK293T cells, and

thus, adjuvant was administered 2 days post vaccination to maximize effect. This study aimed to replicate those findings by comparing the antitumoral efficacy of vaccine and adjuvant delivered simultaneously (Group I) v/s adjuvant given 2 days post vaccination (Group II). Group II females had the smallest tumor sizes (Fig. 1b); on day 23, Group II females had an average tumor size of 161 mm^2 whereas Groups I and III (CpG alone) were 268 mm^2 and 336 mm^2 respectively (Fig. 1a, 1c). Furthermore, in males, there were no clear differences between the two groups – Group II males were euthanized on day 19 and Group I males were euthanized on day 21, and both had similar tumor sizes (Fig. 1e).

Overall, the results from this pilot study seem to align closely with the existing literature. Female mice showed slower tumor growth when compared to males, as seen by longer survival and smaller tumor sizes. Additionally, these results also provide evidence for the marginal role of CpG C in this tumor model when given as a single therapy, as the tumor sizes and survival rates between the no treatment group (Group IV) and adjuvant alone were similar and not significant. Furthermore, the decision to deliver adjuvant 2 days post vaccination was also vindicated by these findings as Group II females displayed the smallest tumor sizes and the longest survival time overall. However, to confirm statistical significance, more experiments need to be performed in the future.

3A2. Age Differences

In addition to examining the role of sex, this experiment also aimed to understand the impact of age in the B16F10 tumor model. It is important to note that this analysis was only performed using female mice. In order to perform this analysis, tumor sizes and survival were measured for the current experiment and compared to a previous experiment. 10-14-week-old female mice receiving vaccine and adjuvant 2 days later were compared to an earlier experiment involving 4-6-week-old female mice receiving the same therapy. In order to maximize consistency during the comparison, only tumor sizes measured on the same days were used; a 2-way ANOVA was performed on the data for analysis and revealed no significant overall differences between the two age groups (Fig. 1f). While limited by sample size, this initial observation was interesting; while some literature has implicated age in slower tumor growth (58), many of these studies have simply evaluated growth in response to no treatment. Since this comparison involved a previous experiment, a no treatment group was not available for comparison – thus, it will be critical to evaluate whether there are differences in tumor size between older and younger mice when not given any treatment. Furthermore, it is also important to understand how our other therapies will influence this observation, and whether older males respond differently compared to younger males; this is especially important as the tumor growth was highly robust in older males, and no significant differences were observed between any of the therapy groups.

CONCLUSIONS

The immune responses underlying these potential differences must also be investigated; Dakup et al (56) indicated that female mice had a higher infiltration of CD8+ T cells compared to males, which was critical for a stronger antitumoral response. However, there are few studies examining these differences – it is important to understand how different therapies (such as chemotherapy and immunotherapy) differentially impact female and male mice. In humans, men are diagnosed with melanoma at a higher rate than women, and also have a slightly higher mortality rate (2,3). Interestingly, data indicates that recent immunotherapies have shown better efficacy in men than women. Both phase I and II trials on ICB showed that while both men and women benefitted from treatment, men showed a significantly greater level of response (120). According to our results, tumors in the older male mice grew faster compared to the females, not allowing enough time for the vaccine to establish anti-tumor immunity. In our model, treatments were given either 5- or 7-days post challenge, but it may be worth investigating whether earlier treatment of males may yield different results; since the tumors grow faster, earlier treatment could potentially allow for the generation of an antitumoral response in time. Furthermore, it would also be important to see if the initial results seen from the ICB therapy trials would apply to our therapy with 5aza and IFNα. Ultimately, there is minimal literature available on the impact that age and sex have on the natural progression of tumor lines such as B16F10, as well as the differences in response to various cancer therapies; it is necessary that more experiments be conducted in order to

further elucidate the observations made in this pilot study. This is especially important as the 10-14-week-old mice used in the study are not completely analogous to the older human population.

3B. Combination Therapy with an Immature DC Targeting Vaccine, IFNα, and 5aza, and its role in TLS Formation

3B1. The Role of CCL19

Previous work in our lab utilizing the same experimental model revealed upregulated levels of CCL19 in all therapy groups, with the highest upregulation seen with vaccine, IFNα, and 5aza therapy. Furthermore, regression analysis revealed a tight correlation (Sup. Fig 1) between high levels of CCL19 and smaller tumor size. CCL19 is a “homing” chemokine responsible for trafficking CCR7+ immune cells and lymphocytes into the lymph node and is important for facilitating productive adaptive immune responses within SLOs. CCL21 also binds CCR7, and as a consequence of binding the same receptor both chemokines have a number of redundant functions involving immune cell recruitment to SLOs. While CCL19 is secreted by mature DCs, CCL21 is primarily secreted by the endothelium of afferent lymphatic vessels, whereas HEVs and stromal cells secrete both. However, a few studies have attempted to outline differences between the two; specifically, it has been noted that CCL19 is the only chemokine known to trigger robust phosphorylation and internalization of the B arrestin receptor, which leads to more effective antigen presenting DC and CD4+ CD8+ T cell migration compared to CCL21 (59-62), as well as possibly being more soluble

and thus more available to the local environment. Many of these functions have been elucidated using *plt* mice, which are deficient in both CCL19 and CCL21, as well as *ccr7-/-* mice. These differences seem to be stressed in our findings as well, since qRT-PCR analysis of tumor tissue revealed no consistent expression pattern or correlation between CCL21 and tumor size (Fig. 2a and 2b). In the context of immune responses however, the differences between CCL19 and 21 remain controversial. While both are homing chemokines, some studies report CCL21 as being more important for T cell and DC migration (63), whereas others confer equivalent roles in DC and T cell activation. This role is especially critical in the context of this study as a higher influx of CD8α+CD11c+ DCs was seen in the combination group (although not significant), and CD8+ T cells were significantly upregulated in the same group that also showed the highest expression of CCL19 – Group III (Fig. 4). Importantly, Marsland et al observed that CCL19 did not directly activate T cells – as seen by the inability of naïve T cells coincubated with CCL19 to become activated – and thus, all T cell proliferation occurred by way of DC maturation. Furthermore, CCL19 also upregulated co-stimulatory molecules CD40 and CD86 as well as cytokines IL-12 and IL-1β in DC, significantly enhancing their ability to engage and activate T cells. DCs activated with CCL19 also preferentially induced Th1 licensure in T cells, as seen by significantly higher levels of IFNγ producing cells compared to a decrease in IL-4 producing cells. Additionally, the activation of DC in *plt* mice was also examined; DCs isolated from these mice showed comparable levels

of MHC II but showed significantly lowered levels of co-stim molecules CD80 and CD86 (63). In a 2011 study by Haessler et al, the differences between CCL19 and 21 regarding their ability to attract DCs in a 3D microenvironment was investigated. Their results indicated equivalent migration of DCs towards both cytokines at small gradients, but more efficient migration towards CCL21 at a higher gradient, as well as when CCL21 and CCL19 were both present in the system (62). In contrast, a more recent study by Hansen et al noted a different finding; they showed that human monocyte derived DCs migrated more potently towards CCL19 than CCL21. Additionally, they concluded that autocrine CCL19 signaling by DCs negatively impacted their migration towards CCL21 with differences in potency being the deciding factor (64). In our model, we see elevated levels of overall CD11c DCs and CD8α+CD11c DCs in our combination group, which is critical since they are a proinflammatory subset of DCs capable of cross presentation and consequently activating both CD4 and CD8 T cells. These findings, together with the observation that CCL19 is a robust DC licensor and that those DCs preferentially activate Th1 T cells, may help explain the robust anti-tumoral response in the combination group.

As noted in the Results, CCL21 expression was not as upregulated as CCL19. These results were surprising since both bind the same receptor and share a multitude of functions. However, reviews focused on CCL21 in melanoma have noted conflicting results (107). In certain studies, CCL21 is shown to have a beneficial role, where its expression in metastatic melanoma

is corelated with a stronger immune response, especially regarding CD8+ T cell recruitment into tumor sites (107-109). CCL21 is also known to activate T cells by upregulating CD4 and CD8 expression and encouraging a Th1 bias in activated T cells (108). Furthermore, multiple studies evaluating TLS in melanoma often report CCL21 overexpression (108). However, in a study by Shields et al., tumor secreting CCL21 in the B16 melanoma model facilitated the development of an immunosuppressive TME by increasing Tregs and MDSCs, whereas CCL21 deficient TMEs promoted greater anti-tumoral immunity (110). Conversely, a group using a DNA vaccine encoding Trp2 in B16 melanoma found that CCL21 was an effective adjuvant that enhanced Trp2 specific immunity; however, timing was critical, and the protocol was only effective when CCL21 was given 24 h prior to the vaccine, at the same site (111). As expected, the tumor specific immunity was dependent largely on cell mediated immunity, which CCL21 is known to stimulate. Similarly, another study examining the role of CCL21 secretion by melanoma cells found that high CCL21 expression via a ubiquitin promoter resulted in massive immune infiltrates of CD4 and CD8 T cells, as well as CD11c+ DCs, and complete rejection of CCL21 positive melanomas within 3 weeks (109). Importantly, in melanoma, the CCL19/CCL21/CCR7 axis is known to contribute to increased metastatic potential, and high expression of CCR7 is associated with poor prognosis (112). In one study, overexpression of CCR7 in B16 melanoma cells increased metastatic potential of the tumor, while neutralizing CCL21 via a specific antibody removed this metastatic ability

(113). Additionally, CCL21 has also been shown to be involved in TLS formation, where many solid tumors presenting with TLS also show an increase in CCL21 expression (46). Since TLS are often correlated with better prognosis, this presents another scenario where heightened levels of CCL21 in the system indicate a strong immune response. Ultimately, there are conflicting data on the role of CCL21 in melanoma. In some situations, CCL21 is associated with immune events that favor a strong antitumoral response whereas in others, its presence is related to a tolerogenic TME and poor prognosis. More research is needed to understand what exactly drives these differing responses and how they can be manipulated to create a strong antitumoral immune response.

3B2. The impact of TILs, DCs, and NK cells

Th1 T cells are key enactors of cell mediated immunity, which has been touted as being critical to an effective anti-tumoral response. Multiple studies across multiple types of malignancies have validated this belief (65-67), and often, a shift from Th2 biased responses to Th1 responses marks an upward trajectory in terms of an effective response – as seen by tumor regression and disease remission (68). CD8+ CTLs possess active killing ability; it is their successful activation which shapes an effective anti-tumoral response (69,70). Our results showed significantly elevated levels CD8+ T cell numbers and higher levels CD8α DCs in our combination therapy model, and their presence correlated strongly with reduced tumor size (Fig. 3 and 4). Additionally, another critical subset of cells that has been implicated as part of

anti-cancer immunity is the CD8+ effector memory subset (Tem). These T cells are characterized by the loss of CCR7 and CD62L and maintenance of CD44, allowing their dissemination from SLOs and trafficking into distant tissues where their presence is required. Importantly, Tem cells possess lytic activity and can kill cancerous cells; they have reduced activation requirements compared to their naïve counterparts and persist for a long time (71). Increased levels of CD8+ T cells and CD8 memory T cells have been correlated with positive prognosis in multiple cancers (72-74), and our results confirm these observations as both effector CD8 T cells and CD8 Tem were highly correlated with a reduced tumor size (Fig. 4d). In a previous experiment, the presence of CD4 T cells was also evaluated, but the numbers remained relatively unchanged over the different treatments (Supp. Fig 2) and were not correlated with a reduction in tumor size.

Unlike T cells however, B cells did not show the same trends. Surprisingly, the CD19+ B cell population was highly reduced in the combination therapy group and showed no correlation with reduced tumor size (Fig. 4e and f). Multiple studies over the years have delineated the anti-tumoral role of B cells in the B16F10 melanoma model, and studies in human melanomas have paralleled the same findings (75-77). Interestingly, in one such study, B cell depletion in mice using an anti-CD20 antibody resulted in significant impairment of Tem and Teff populations (78); however, in our model, we saw enriched populations of both CD8 T cells and CD8 Tem cells in the combination group, which had a depression in CD19+ B cell counts. This

decrease in B cells was noted over two experiments (Fig. 4e and Supp. Fig 3) and the same trend was noted in both. In addition to CD19, B220 was also utilized as a pan B cell marker – the same trend was noted with B220+ CD19+ B cells (Fig. 4e). It is important to note that most studies focused on identifying B cells in TLS use the marker CD20 as opposed to CD19; while there is no reason to suggest that CD19 expression can be downregulated on B cells, future work will focus on utilizing a CD20 marker instead. Overall, our results present an interesting contrast to the literature as our combination therapy model shows enhanced survival without significant CD19+ B cell infiltration and seems to depend on T cell and DC infiltration instead.

Since there was an enrichment of B220+ cells in the combination group that did not correspond to B cells, flow cytometry using anti-CD11c, anti-Gr-1, anti-NK1.1, anti-B220, and anti-CD35/21 was performed to further classify the B220+ and B220- CD19- subset. Results revealed enrichment of an NK population (NK1.1+) in the combination group that was tightly correlated to reduced tumor size (Fig. 4g and h), as well as an upward trend in plasmacytoid DCs (pDCs) (B220+Gr-1+CD11c) between the vaccine and combination groups ($p = 0.0972$, Fig. 3a). Both NK cells and DCs have been implicated in anti-tumoral immunity, but research regarding pDCs is has produced conflicting results (79,81). In melanoma, pDCs often promote an immunosuppressive environment – a function which contrasts with their ability to preferentially activate Th1 T cells via production of type 1 IFN, as well as their ability to promote NK cell activation and development of mature DCs

(79). Importantly, while their presence in melanomas has historically been correlated with poor patient outcome, regressing tumors have enhanced presence of activated pDCs capable of producing type 1 IFN, which is a key function that is lost in pDCs isolated from progressing melanomas (79,80). Furthermore, pDC activation occurs via the engagement of TLR7 and TLR9, and stimulation with their agonists has shown to induce type 1 IFN production by pDCs present in the TME – as seen by the response generated by topical application of imiquimod to superficial basal carcinomas (81). This finding was replicated by the intratumoral injection of CpG-ODN (a TLR9 agonist) into murine melanoma, where systemic T cell immunity was dependent in part on successful pDCs activation (82). This observation was furthered by the fact that intratumoral injection of “blood-derived CpG-activated pDCs” successfully elicited NK cell and cDCs activation, which were then critical to the induction of tumor specific T cells (82). These findings may be highly relevant in the context of our model as we injected IFNα intratumorally (if indicated) (and intramuscularly in a previous publication, which showed similar effectiveness) as a series of 1 high dose followed by 3 low doses, as well utilizing CpG C as our vaccine adjuvant. CpG C is a potent TLR9 agonist known to stimulate pDC activation both *in vitro* and *in vivo*; this combination may be critical in providing a boost to poorly activated pDCs, which could then engage in the productive cross talk of activating T cells – a phenomenon we observe potently in the combination group. While results from this experiment did not

indicate significant differences in pDC levels between groups, these findings warrant further study.

NK cells also form a critical component of this antitumoral immune scaffolding; these cells play an essential role in the immunosurveillance regarding cancer, and their lethal activity is often inactivated by cancer cells using a wide variety of mechanisms (83-85). Furthermore, NK cell infiltration into tumors has been correlated with better prognosis as well (86). Activation of NK cells often occurs by way of type I IFN; *in vitro* studies have shown NK cell activation as a result of cytokines such as IFNα and IL-12 secreted by mature DCs (87,88), and reciprocally, activated NK cells are also capable of activating DCs and augmenting tumor specific T cell responses (89,90). This is especially important in our model as NK cells, like pDCs, can also be activated by type 1 interferons and/or TLR7/TLR9 agonists. Since we see enriched populations of both NK cells and DCs in the combination group that are correlated with a reduced tumor size, it is highly possible that this crosstalk plays a critical anti-tumoral role in this system – especially since these productive, bidirectional responses are tightly involved with T cell activation (91) and may involve colocalization of DCs and NKs. Interestingly, there is also evidence that a strong presence of DC-NK cell crosstalk may be enough to bypass the role of CD4 T cells in CTL induction (92). This may represent an important finding, as in our model, CD4 T cell counts were not significantly upregulated in any group and did not show the same trends as either CD8 T cells, NK cells, or DCs.

3B3. Potential TLS formation

In addition to its role in DC migration, maturation, and subsequent T cell activation, the CCR7/CCL19/CCL21 axis is critical to both maintenance and function of SLOs; thus, its possible role in the formation and function of tertiary lymphoid structures has also been hypothesized (40,42). TLS are lymphoid aggregates resembling SLOs that form ectopically and have been observed in a multitude of pathologies including infectious diseases, autoimmune disorders, and cancer (40-42). They share significant similarities in both structure and function to SLOs, including the presence of APCs such as DCs and macrophages, as well as B and T cells. Because CCL19 is responsible for homing CCR7+ immune cells into the lymphoid follicle, its expression has been consistently noted in solid tumors displaying TLS (42,46); furthermore, when compared to tumors without TLS, CCL19 expression is significantly upregulated in those tumors displaying TLS as confirmed by IHC. Although TLS function in cancer is a continuously evolving topic of research, many studies across different types of cancer seem to agree that the presence of TLS in solid tumors presents a prognostic advantage (40-46). Since our results indicated a combination of factors involved in TLS (increased T cells, DCs, and CCL19 expression), we focused on identifying other factors strongly associated with TLS formation in melanoma. Like CCL19, another chemokine that is upregulated in tumors having TLS is CXCL13. CXCL13 binds its cognate receptor CXCR5, and is involved in B cell trafficking to the SLOs as well as germinal center

organization (114). In a study by Henry et al., CXCL13 blockade by antibody disrupted B cell organization within TLS, and another study focused on immunotherapy in melanoma (115) also noted that tumors presenting with TLS had upregulated expression of CXCL13. This same study also noted upregulated levels of CCL19 in TLS. Additionally, another critical component of TLS are high endothelial venules (HEV), which are distinct blood vessels that allow the entry and exit of lymphocytes from the periphery and into TLS, where they can interact with APCs and become activated (43,49). Lymphocytes express CD62L (also known as L-selectin), which binds the peripheral node addressing receptor (PNAd) present on the HEV. This interaction allows the extravasation of B and T cells and their subsequent interaction with APCs. In one study, the density of HEV was correlated with better patient outcomes in melanoma (116), in line with findings regarding TLS in melanoma also being a positive prognostic factor (56,38,42). Thus, based on these findings, we tested the tumor lysate for both CXCL13 and CD62L expression. As seen in Fig. 3d, when compared to the IFN+5Aza group, CXCL13 expression was significantly upregulated in the combination therapy group, and so was CD62L. Interestingly, for CXCL13, based on the ΔCt values, there were no significant differences between the combination group and vaccine only. This is most likely due to the high variance seen in the vaccine only group, whereas in the triple therapy group, the values were relatively clustered together. Another interesting thing to note is that CXCL13 is primarily involved in B cell recruitment to SLOs/TLS, but in our model, we

saw a significant downregulation of CD19+ B cells in our triple therapy group. In recent findings, however, CXCL13 has also been involved with T cells (114). Specifically, in a study by Silina et al. that focused on lung cancer, tumors with high CXCL13 expression were correlated with increased recruitment of CXCR5+ T cells into TLS, and better patient outcome (117). Another study reported that CXCL13 secretion induced by lysophosphatidic acid (LPA) resulted in increased CD3+ cell influx, which was ablated using a CXCL13 neutralizing antibody (118). Thus, these findings suggest that CXCL13 also has a critical role in T cell recruitment in addition to its role in B cell recruitment. In our model, CXCL13 expression was significantly upregulated in the triple therapy group, and this upregulation was highly correlated to reduced tumor size (Fig. 3e). Similar to CXCL13, CD62L expression in the combination group was also upregulated, albeit to a lesser extent, and there were no significant differences between vaccine and the triple therapy group (Fig. 3e). However, based on the ΔCt values, differences between IFN+5aza and the combination group were significant. As mentioned, CD62L on lymphocytes binds PNAd on HEV, which form a critical component of TLS. In our model, mice receiving combination therapy showed a significant increase in both CD3+CD8+ T cells, as well as Tem cells. While Tem cells have downregulated CD62L expression, naïve T cells and central memory T cells express CD62L. Our flow data indicated that a majority of T cells in the combination group are effector memory T cells, which are capable of leaving SLOs and mounting an immune response at the tumor site, but the

overall increase in CD8+ T cells in the combination therapy group correlates with the increase in CD62L expression seen in the same.

CONCLUSIONS

Combined, our current and previous data show an increased influx of DCs, NKs, and T cells, as well as upregulation of CCL19 and CXCL13. Thus, based on these findings, the mechanism of increased survival in the combination group may be through the formation of TLS. However, the studies performed are not sufficient to prove TLS presence and future studies will focus on building on these data to arrive at a more conclusive answer.

Chapter 4

Future Directions

4A. Age and Sex as Variables in the B16F10 Murine Melanoma Model

The murine B16F10 model is one of the most widely utilized models of melanoma in oncology research, and yet its implementation has primarily been limited to female C57BL/6 mice. There is a dearth of literature available on this matter, and so, this pilot study aimed to investigate both age and sex differences using a previously published experimental model involving a unique vaccine platform. Our results showed largely what the few published articles in the field have reported, especially regarding sex differences. Female mice lived longer and had overall slower tumor growth whereas tumors in male mice grew very robustly and remained largely unresponsive to treatment. It is important to note that these differences were limited to observation, and the power of the experiment was limited. For the future, it will be critical to repeat these particular experiments and possibly incorporate other scenarios as well. For example, this study did not compare age differences in male mice or differences in natural progression of the tumor between older and younger female mice. Additionally, it will be critical to parse the immunological phenomena governing these differences. Finally, as mentioned, 10-14-week-old mice are analogous to 30 years of age in humans, which is not considered old. Thus, in order to make appropriate comparisons between older mice and older human beings, the mice used in the study would have to be over a year old.

4B. Combination Therapy with an Immature DC Targeting Vaccine, IFNα, and 5aza, and its role in TLS Formation

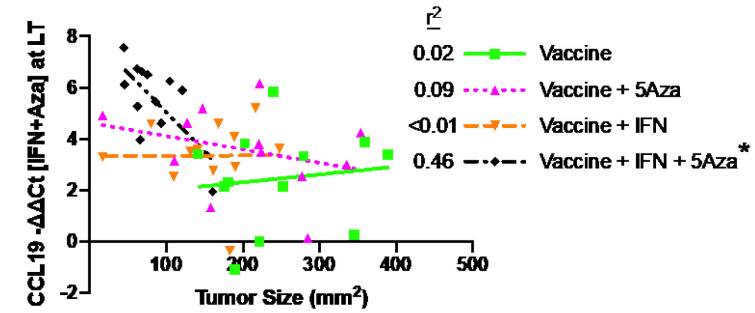
Our unique combination therapy model involves administering a MIP-3α fused DNA vaccine, IFNα, and 5aza therapeutically post lethal subcutaneous challenge with B16F10 melanoma cells. In this group, we see significantly enhanced survival as well reduced tumor growth when compared to mice treated with either vaccine alone or only IFN+5Aza. In order to enhance our knowledge of an effective antitumoral response, it is critical to parse the immune interactions facilitated by the combination therapy; thus, this study aimed to understand and expand upon the cellular makeup of the TME and investigate whether the augmented survival could potentially be dependent on the formation of tertiary lymphoid/tertiary lymphoid - “like” structures. Our results corroborated previous findings and presented new data as well. Gene expression data also showed significantly upregulated levels of CXCL13 in the triple therapy group, another marker implicated in TLS formation. However, expression was only analyzed for one experiment, and in order to fully understand the significance of this observation, more experiments will be tested for expression. Additionally, while CD62L was used to approximate HEV expression, a PNAd marker will be used in the future to increase accuracy as PNAd is actively expressed on HEV. This experiment highlighted the critical role of CD8+ Tem cells in controlling tumor growth, as well as highlighting the possible critical role of NK-DC crosstalk in this particular therapeutic model. Furthermore, our work indicates the possibility of an effective antitumoral response without the presence of tumor infiltrating B cells,

and a focus instead on T cells, NK cells, and DCs. Future experiments will focus on classifying the enriched NK cell population in order to fully understand their role in the system, and staining the tumor tissue for presence of TLS, via markers such as CD3 and CD11c. We will also stain tumor tissue with a CD20 marker for B cells to compare any possible differences between CD19 and CD20 expression. Most publications staining tumor tissue for TLS utilize CD20 as a B cell marker, thus it will be important to confirm that we truly do not see B cells in our tissue, as opposed to a possible issue with CD19 expression on B cells.

Chapter 5

Supplementary Figures

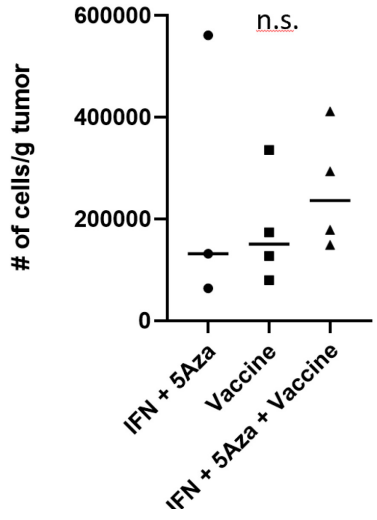
1.



overall r2: 0.195, p = 0.002

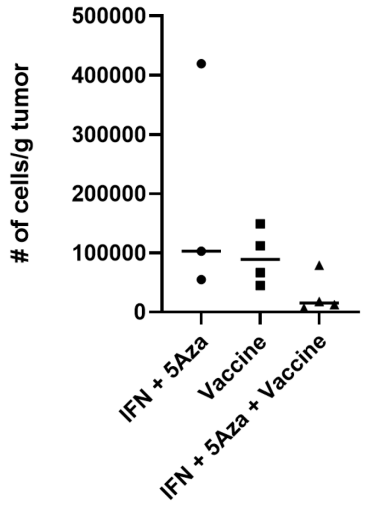
Supp. Fig. 1. **Correlation analysis between CCL19 -ΔΔCt levels and tumor size (mm²).** Starred group represents the triple therapy/combination therapy group.

2.



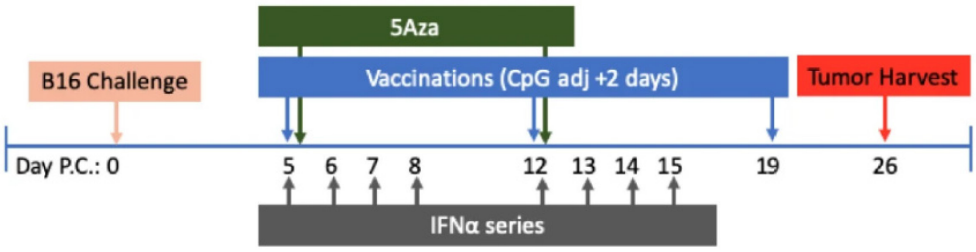
Supp. Fig. 2. **Effect of different treatments on CD4 expressing t cells/g of tumor**. Flow data showing number of CD3+CD4+ T cells per g of tumor.

3.



Supp. Fig. 3. **Effect of different treatments on CD19 expressing B cells/g of tumor**. Flow data showing number of CD19+ B cells per g of tumor.

4.



Supp. Fig. 4. **Timeline of the experiment.**

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